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AUGUST, 1990

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THE
BIOLOGICAL BULLETIN

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INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts outstanding original research reports of general interest to biologists throughout the world. Papers are usually of intermediate length (10–40 manuscript pages). Very short papers (less than 9 manuscript pages including tables, figures, and bibliography) will be published in a separate section entitled "Notes." A limited number of solicited review papers may be accepted after formal review. A paper will usually appear within four months after its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts that do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in no smaller than 12 pitch, using double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Please, no right justification. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978) and to American spelling. Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Title page.*** The title page consists of: a condensed title or running head of no more than 35 letters and spaces, the manuscript title, authors' names and appropriate addresses, and footnotes listing present addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations.

3. ***Figures.*** The dimensions of the printed page, 7 by 9 inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

4. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables, with their headings and footnotes, should be typed on separate sheets, numbered with consecutive Roman numerals, and placed after the Literature Cited. Figure legends should contain enough information to make the figure intelligible separate from the text. Legends should be typed double spaced, with consecutive Arabic numbers, on a separate sheet at the end of the paper. Footnotes should be limited to authors' current addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations. All such footnotes should appear on the title page. Footnotes are not normally permitted in the body of the text.

5. ***Literature cited.*** In the text, literature should be cited by the Harvard system, with papers by more than two authors cited as Jones *et al.*, 1980. Personal communications and material in preparation or in press should be cited in the text only, with author's initials and institutions, unless the material has been formally accepted and a volume number can be supplied. The list of references following the text should be headed Literature Cited, and must be typed double spaced on separate

pages, conforming in punctuation and arrangement to the style of recent issues of *The Biological Bulletin.* Citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS (BIOSIS List of Serials; the most recent issue). Foreign authors, and others who are accustomed to using THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K.) useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond., Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, page proofs, and charges.*** Authors receive their first 100 reprints (without covers) free of charge. Additional reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date. Authors (or delegates for foreign authors) will receive page proofs of articles shortly before publication. They will be charged the current cost of printers' time for corrections to these (other than corrections of printers' or editors' errors). Other than these charges for authors' alterations, *The Biological Bulletin* does not have page charges.

The Marine Biological Laboratory

Ninety-Second Report for the Year 1989
One-Hundred and Second Year

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Prosser Gifford, *Chairman of the Board of Trustees*
Harlyn O. Halvorson, *President of the Corporation and Director*
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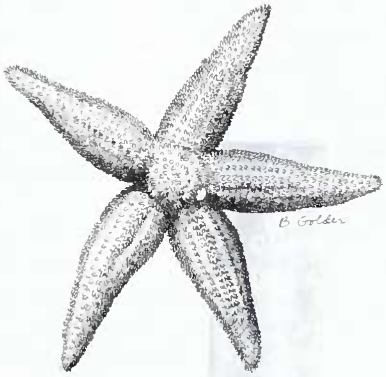
Kathleen Dunlap, *Clerk of the Corporation*

Contents

Report of the President and Director 1
Report of the Treasurer 6
Financial Statements 8
Report of the Librarian 18
Educational Programs
    Summer Courses 21
    Short Courses 26
Summer Research Programs
    Principal Investigators 30
    Other Research Personnel 32
    Library Readers 35
    Institutions Represented 36
Year-Round Research Programs 40
Honors 46
Board of Trustees and Committees 49
Laboratory Support Staff 53
Members of the Corporation
    Life Members 55
    Regular Members 56
    Associate Members 69
Certificate of Organization 73
Articles of Amendment 73
Bylaws 74



Report of the President and Director

If you spend a few hours in the stacks overlooking Eel Pond, leafing through old directors' reports in the bound volumes of *The Biological Bulletin*, you might learn any number of lessons—about science in our century, about institutions and governance, about the MBL, that odd biological experiment in which scientists have tried, sometimes against long odds, to run their own institution, a seaside laboratory where they and their intellectual descendants can practice science as it ought to be practiced.

Among other things, you might notice how little some things have changed over the years at the MBL. The successes we've enjoyed and the problems we've encountered—both have remained surprisingly constant over the decades. That observation itself has periodically reappeared in the August supplement to the *Bulletin*, where various directors have noted how unchanging our successes have been, how eternal our needs—especially the need for better financial support.

It is interesting, and instructive, to hear a succession of directors assuring readers that the MBL is still fulfilling C. O. Whitman's vision. For most of the 20th century, our directors have been reporting that the Laboratory still serves as a congress of American biology, still draws excellent people from excellent institutions, still spawns first-rate research and instruction, and still spans a wide range of biological interests—including emerging interests. Acknowledging that the population of American biologists has grown by orders of magnitude and a smaller percentage of the best biologists can be accommodated by any one institution, I can nonetheless report that, once again, it is still true that the MBL is hosting excellent investigators and simultaneously training some of the best young biologists, many of whom will no doubt rise to the top of their fields.

It is also interesting, and instructive, to hear directors reporting over the decades on continuing—or recurring—problems, many of which, I must also report, are still with us. Across the decades, various directors and long-range planning committees have written of our need for a larger endowment, increased support for our courses, more affordable housing for young investigators, and improved research facilities for all, including better collecting and holding facilities. Those needs are all still facing us, but here I want to address the last and foremost. Simply put, we must improve our research facilities if the MBL is to stay at the forefront of American biology.

Marine Resources Center, 1924–1989

Most immediately, we need a new Marine Resources Center to provide investigators and students with a reliable, healthy, and genetically defined supply of marine organisms.

Our current holding facility was erected in 1924; the call to improve the facility—then known as the Supply Department—began appearing in these pages 50 years ago, when the 65-year-old building was just 15 years old.

By the close of the 1930s, an *ad hoc* committee of the Corporation had declared the holding facilities obsolete.

In 1940, Associate Director Charles Packard urged an improvement in Supply Department facilities, citing a 1939 report of the Committee on Policies and Future of the Laboratory. Sketches of a replacement structure apparently were drawn up, but the work was never done.

The current wooden building underwent modest renovations in the 1950s. By 1970, a new long-range planning committee was calling for a new Marine Resources Building. The new facility played a key role in a major proposal to the NSF. In his annual report for 1971, Director James Ebert described the need for a building "embodying environmentally controlled facilities suitable for the development of genetic strains of selected marine species and to initiate long-term studies of the influences of changing environmental conditions on their life histories and behavior."

By 1976, an *ad hoc* committee on marine resources had worked with architects to design a three-story, 35,000-square-foot replacement for the old Supply Department. Director Keith Porter reported in these pages that the proposed new building would include "controlled systems for animal maintenance and an improved salt water system for uninterrupted flow during maintenance of filter beds." The second floor would be "devoted to the culture of marine forms (involving) the long-term maintenance of young and adult organisms and the controlled propagation of organisms to provide genetic stocks and certain species that are either scarce . . . or difficult to obtain." The top floor would be devoted to "research in genetics and the pathology of marine organisms."

The following year, James Ebert (who had returned to direct the Laboratory) reported a move to develop a Marine Resources Center that could provide laboratory space for research and training programs in genetics, development, pathobiology and the pharmacology of marine organisms.

In 1979, planning for the new facility continued when the building was included as a central component in the Second Century Fund Campaign. A detailed feasibility study had been carried out by 1983, and in his 1985 director's report Paul Gross discussed the Laboratory's needs with his usual eloquence: "I noted earlier," Gross wrote, "that the MBL's needs of today would not surprise C. O. Whitman, were he alive: he would understand perfectly that the MBL must, if it is to survive as a headlight, rather than a taillight, of biology, practice and teach the most advanced biology, no matter where it leads, no matter what biological materials are used for experimentation, no matter how much money it costs."

First among the urgencies Gross called to the Corporation's attention, if the MBL was to remain a headlight of biology: "A new Marine Resources Center, without which, sooner rather than later, the provision of research specimens will fall into decline, research on their laboratory culture and genetic definition will stop, and the year-round program in general will be brought into disarray."

In 1987, a Committee on Long Range Goals, chaired by Gerald Fischbach, offered to the Trustees, among other recommendations, this by-now-familiar caution: "The dilapidated Marine Resources Building, constructed in 1924 and last renovated significantly after the 1953 hurricane, will soon undermine the MBL's position as a national center for studies of marine organisms."

Finally, our proposal for a Marine Biomedical Institute for Advanced Studies (MBIAS), compiled in our centennial year of 1988, includes a 32,000-square-foot building for the year-round breeding and maintenance of important marine species. The proposal identifies a state-of-the-art Marine Resources Center as an absolutely essential feature of any marine institute that seeks to play a major role in our national biomedical research effort.

I offer this historical tour of directors' reports and planning committee findings to remind the Corporation that the logic for building a new Marine Resources Center is compelling.

In the last two years, we have made much progress toward the necessary building, but much hard work remains. We have to raise more construction funds. We have to have additional discussions with the local community and officials. And we have to do more careful analyses of how we will cover the new facility's operating costs. These tasks are now before us; as I write this report, in the spring of 1990, much of the administrative staff and many Trustees and friends of the Laboratory are wrestling with these difficult matters.

But while there is much work left to be done before the long-awaited Marine Resources Center is up and operating, let me remind you that it is closer now than it has ever been before. By the end of 1988, the Trustees had approved the idea within the larger MBIAS proposal, which also includes building a new laboratory building. And the federal government had responded with a $2.2 million appropriation toward planning and construction of the two buildings. Additionally, Congress voted and President Reagan signed an authorization for such funds as necessary for construction of the MBIAS. The authorization, of course, does not guarantee full funding, but it does signal Congress' approval and allows us to go back for additional appropriations in subsequent years with some reasonable hope of success.

In 1989, we were awarded some of those additional funds in the form of a $2 million appropriation. We now have raised $4.2 million toward the planning and construction of MBIAS facilities. We proceeded in 1989 to draw up plans for the new Marine Resources Center. (Simultaneously, we were making plans for the Advanced Studies Laboratory.)

In planning the Marine Resources Center, we worked with the Falmouth Town Planner, the Building Commissioner, and other department heads of Falmouth town government. In August of 1989, we put models on display in Swope for MBL Trustees and Corporation members. Throughout the year, we shared our building plans with various local groups, including the Conservation Commission, the Falmouth Chamber of Commerce, the Falmouth Historical Society, the Woods Hole Community Association, and our own Falmouth and Woods Hole Advisory Committees. In December, we formally applied to the town for a building permit. (The permit was approved 5 January 1990.)

At the close of 1989, we can entertain realistic hopes that this important building will become a reality before too many more directors' reports are filed.

Research

Research in 1989 proceeded along some established MBL lines and in some new directions, as well. The Ecosystems Center had an outstanding year, financially as well as in the field and press. Among the many papers that appeared from the Ecosystems group, one particularly widely read paper on methane uptake in temporal forests appeared in *Nature* and was promptly reported by a bevy of journalists. That paper suggested a link between acid rain and the greenhouse effect, a finding that caught the attention of ecologists around the world. On an administrative note, Dr. Jerry Melillo was promoted to co-director of the Ecosystems Center. He now shares leadership for that dynamic group with Dr. John Hobbie, who returned in 1989 from a sabbatical year in Sweden.

Dr. Mitchell L. Sogin's new Center for Molecular Evolution arrived in the spring of 1989. Dr. Sogin is also the director of our Workshop on Molecular Evolution, which was held for the second time in 1989.

Dr. Robert E. Palazzo opened a year-round laboratory to study the biochemical regulation of cellular events during meiosis and mitosis. Before receiving his current five-year grant, Dr. Palazzo worked at the MBL as a summer investigator in Dr. Lionel Rebhun's University of Virginia laboratory.

In 1989 we concluded a new five-year financial agreement with Boston University on the Boston University Marine Program. The new agreement, which coincided with BUMP's twentieth anniversary at the MBL, is the first long-term agreement we've reached with BU, and it establishes a more reliable base from which all parties can make their plans about space and resources.

The University of Pennsylvania made a dramatic new commitment to the Laboratory of Marine Animal Health with the establishment of a chair for the new director of that laboratory. Dr. Donald Abt, a veteran MBL scientist and University of Pennsylvania marine epidemiologist, took on leadership of the lab and assumed the Robert R. Marshak Term Professorship of Aquatic Medicine and Pathology.

Together with the Woods Hole Oceanographic Institution, we opened a new facility for molecular biology, including nucleic acid analysis, gene sequencing, and recombinant DNA work. Technical manager for the facility is Dr. Richard Ridge.

The re-initiated Steps Fellowship program provided support for seven junior faculty member investigators in 1989. This important program helps bring new generations of biologists to the MBL at a time when the laboratory can have the most beneficial effect on their careers.

Instruction

The demand for MBL summer courses was high in 1989; we had a 39% increase in applicants over 1988. This year also marked the second successful year for two August courses, Methods in Computational Neuroscience and Workshop on Molecular Evolution, about which we are very enthusiastic.

Certain long-standing problems in our $1.7 million educational enterprise must soon be faced by the Corporation. Most fundamentally, where will we get the funds to continue operating at our present level? We should note that the MBL is not alone in being hit by cuts in educational grants. Nor are we alone in having to answer tough questions put forth by review committees about the value of advanced training at our site. Simultaneously, we are facing the prospect of changing priorities by corporations and foundations that have funded MBL courses in the past.

I believe that we must turn to the university graduate programs that depend on the MBL for advanced training to help us stabilize our federal funding for training and, at the same time, ask them to pay more of the costs of sending their students to the MBL. Simultaneously, we must begin to build an endowment for all MBL courses. This initially will take the form of course-specific endowment funds contributed by course alumni.

Other major questions concern the direction and organization of the courses. I hope Corporation members will work with us to define the priorities and seek the support to continue what is still a superb educational program at the MBL.

The Zeiss School of Microscopy opened at the MBL in November. One of our own Trustees, Dr. Dieter Blennemann, president of Carl Zeiss, Inc., is responsible for establishing the new school, which offers week-long courses in microscopy for Zeiss personnel and for other

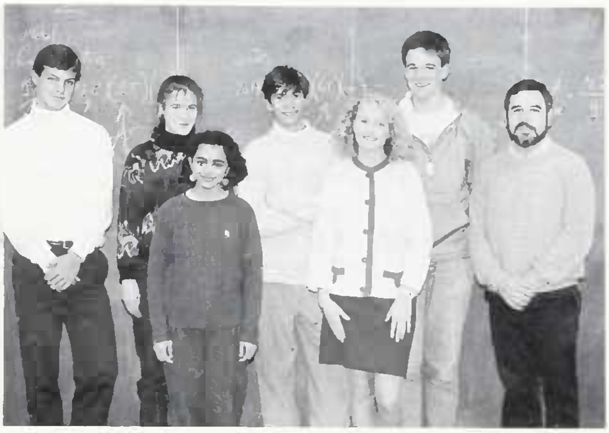
Zeiss School of Microscopy director Philip Presley with student.

academic and industrial users of sophisticated microscopy. Zeiss representative Philip Presley serves as director of the new school.

In a non-traditional educational venture, we hosted our first ELDERHOSTEL session in 1989. ELDERHOSTEL programs are week-long courses for adults 60-years-old and older. Three additional sessions are planned for winter months in 1990.

We also hosted or participated in a number of programs for high school students. With Simon's Rock of Bard College, we hosted an NSF-funded summer research experience for young scholars. The program brought 30 tenth and eleventh grade students to the MBL in August for internships in ecological and environmental science. Our own Futures in Science (FiS) program continued with four Falmouth High School students participating in 1988–1989 and six in 1989–90. Futures in Science is funded and administered by the MBL Associates.

ELDERHOSTEL at the MBL. From left to right: instructor and MBL Corporation member Maurice Sussman, ELDERHOSTELer Mary Spahr, and Chairman of the ELDERHOSTEL Board Eugene Mills.



The 1989 FiS Kids. Left to right (back): Sam Trumbull, Jennifer Shepard, Brendan Cowan, and Elijah White; (front): Supritha Rajan, Tammy Jackson, and Ted Rowan (teacher/advisor).

In the summer of 1989, we joined with a consortium of other science institutions, businesses, and local educators to form the Woods Hole Science and Technology Education Partnership. Still in the planning stages at the close of the year, this partnership is an effort to develop science programs and activities for local high school students.

Library

We have made exciting progress in upgrading our library services, as Director of Library Planning Catherine Norton describes in her library report. MBL scientists can now access a host of databases and tools through OSIS, the library's on-line scientific information system. We are putting in place a village-wide network to link scientists' in-lab computers to the library. Through the network, individual scientists will also be linked to each other and to the outside world. This modernization of library services is the result of recent gifts and grants from the A. W. Mellon Foundation, the Bay Foundation, and the Howard Hughes Medical Institute.

Governance

Governance is another subject that echoes through past directors' reports. Changes in the Board of

Trustees, new planning and oversight committees—the MBL seems to be always in the process of trying to figure out how a truly democratic laboratory can govern itself. In 1989, we continued to strengthen our corporate and foundation representation on the Board of Trustees. Two lay Trustees—now officially known as At-Large Trustees—served with distinction on the 1989 Executive Committee. D. Thomas Trigg and Irving W. Rabb, both Trustees of the class of 1990, brought a new level of business sophistication and financial acumen to the Executive Committee.

The Year-Round Scientific Forum continued to be active in 1989. A four-member Council of Year-Round Scientists, elected by Forum members, worked to further define issues and implement ideas raised by Forum members. The Council is advisory to the director. The 1989 Council addressed the issues such as office space for year-round scientists, hiring and promotions policies, grievance procedures, and the need to recruit new young year-round scientists. Council members in 1989 included Drs. Jelle Atema, Anne Giblin, Felix Strumwasser, and Ete Szuts. In May, Drs. Lionel Jaffe and Gus Shaver replaced Drs. Giblin and Szuts.

Personnel

In the autumn of 1989, a group of employees of the Laboratory asked the National Labor Relations Board to supervise an election to determine whether Local 767 of the Hospital Workers Union would represent MBL maintenance, clerical, and some technical employees for the purposes of collective bargaining. The Executive Committee directed the administration to carry out an informational campaign, in order that MBL employees would be able to make an informed decision on this important question. The election was scheduled for February of 1990. The events of 1989 provided the occasion for management self-evaluation and for some needed training of supervisors. At the close of 1989, I felt that whatever the outcome of the election, our management team was better off for having undergone a fairly rigorous evaluation and training.

The Biological Bulletin

Dr. Michael J. Greenberg began a five-year term as editor of *The Biological Bulletin* in August, succeeding Dr. Charles B. Metz, who served in that post for many years. Dr. Greenberg is a long-time MBL Corporation member, a former course director, and a veteran of the *Bulletin*'s editorial board. Upon accepting the editorship, he announced a series of initiatives to increase the *Bulletin*'s visibility and appeal to contributors and subscribers, including the use of color and glossy covers and a new Research Notes section in *Nature* format. He also established *The Biological Bulletin Board*, a periodic newsletter.

National Association of Marine Laboratories

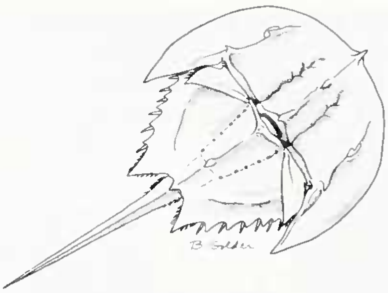
In the autumn of 1989, we joined with about 40 other coastal laboratories to form the National Association of Marine Laboratories (NAML). The association was born out of a growing sense that marine and freshwater labs need to work together on common problems, including the limited pool of federal money available for marine programs, facilities, and training. I was chosen to serve for the next two years as president of the association, and MBL year-round scientist Dr. Alan Kuzirian was elected secretary/treasurer (those appointments will go into effect in 1990).

The new association will officially begin operations 1 July 1990, but by the close of 1989 a NAML delegation was preparing to travel to Washington to make a presentation to NIH directors on the use of marine animals as models in biomedical research.

The Falmouth Forum

Finally, in a combined educational and community outreach effort, a group of volunteers established a winter Friday lecture series titled The Falmouth Forum. Supported in part by the MBL Associates, The Falmouth Forum was designed to bring first-rate lectures and panel discussions to the MBL and Cape Cod communities. The Forum began in November with *Boston Globe* science columnist Chet Raymo giving a presentation on "the soul of science." In December, Falmouth physician E. Langdon Burwell led a panel discussion on America's health care system. The initial presentations were well attended and well reviewed by local audiences.

—Harlyn O. Halvorson



Report of the Treasurer

The year 1989 was a challenging yet successful one for your Laboratory. In the aggregate our fund balances increased from $21.4 to $24.1 million. This growth was due primarily to gifts to endowment and the excellent investment performance of our endowment funds. In addition to the growth in our fund balances, we received a large installment of funds from the Hughes gift. The unspent balance of those funds is now recorded as "deferred support" on the balance sheet. That $4.5 million dollars of deferred support assures the continued strength of the Laboratory's educational program for the next 6 years.

Our current unrestricted fund ended the year with an excess of revenues over expenses of $129,321, of which $78,692 is from the Housing Auxiliary Fund. We were able to make a modest contribution to the Housing Repairs and Replacement Reserve of $49, 144.

The significant events and financial trends evident in 1989 are as follows.

Debt refinancing

In October we refinanced our mortgage note with the Falmouth National Bank with tax-exempt bonds issued by the Massachusetts Industrial Finance Agency (MIFA). This should significantly reduce the carrying cost of the debt on the Memorial Circle cottages and allow us to more adequately provide funds for the repair and replacement needs of our housing stock. This benefit comes with the additional obligations of maintaining a sound financial course and adequate financial reserves and imposes on us an externally monitored requirement for continued financial discipline.

Expanding research base

In 1989 the financial base of MBL-sponsored research rose by 25%—from $3.9 to $5.0 million—continuing a trend begun the previous year. This is a positive and healthy development for the Laboratory. The increase is in large part attributable to the continued scientific success of the Ecosystems Center and to the establishment of the Molecular Evolution Laboratory.

Private gift support

As we negotiated the refinancing of our debt, we again recognized how critical private and foundation gifts and grants are to the financing of the Laboratory's science. While our record is impressive, it points to the continuing challenge we face: renewed and continued private support is vital to our scientific and financial future.

Course support

Although we effectively managed our course budgets for 1989, funding problems loom in the future. Federal funding of instruction is diminishing, reflecting a major shift in funding emphasis and a continued general pressure on the federal budget for science. In 1990 we are experiencing a significant drop from the level of federal support of 1989. Even with multi-year support from the Hughes and MacArthur foundations, this decline in federal support will require serious decisions about course expenditures and future financing.

Planning for new construction

The year 1989 marked the beginning of the preconstruction phase of the new construction projects. Approximately $580,000 of planning and architectural expenses were incurred. We are required by accounting rules to capitalize these expenditures in the anticipation of future construction. We have instituted a Long-Range Financial Planning Committee of the Trustees to evaluate the financial plans for the Marine Resources Center and the Advanced Studies Laboratory. The

Committee will examine the feasibility of plans for financing the construction and the operations of the proposed new buildings. Building the financial support for these new endeavors is a major fund raising and management challenge for the entire MBL community.

The Trustees have asked the Long-Range Financial Planning Committee not to limit themselves to issues connected with the proposed new construction program, but to consider all questions related to the continued financial strength of the Laboratory.

The input of this Committee will strengthen the ability of your administration to plan for and manage the challenging task of maintaining the Laboratory's position at the center of biological science in America.

—Robert D. Manz

Financial Statements

Coopers
&Lybrand

certified public accountants

REPORT OF INDEPENDENT ACCOUNTANTS

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have audited the accompanying balance sheet of Marine Biological Laboratory as of December 31, 1989, and the related statement of support, revenues, expenses and changes in fund balances for the year then ended. We previously examined and reported upon the financial statements of the Laboratory for the year ended December 13, 1988, which condensed statements are presented for comparative purposes only. These financial statements are the responsibility of the Laboratory's management. Our responsibility is to express an opinion on these financial statements based on our audit.

We conducted our audit in accordance with generally accepted auditing standards. Those standards require that we plan and perform the audit to obtain reasonable assurance about whether the financial statements are free of material misstatement. An audit includes examining, on a test basis, evidence supporting the amounts and disclosures in the financial statements. An audit also includes assessing the accounting principles used and significant estimates made by management, as well as evaluating the overall financial statement presentation. We believe that our audit provides a reasonable basis for our opinion.

In our opinion, the financial statements referred to above present fairly, in all material respects, the financial position of Marine Biological Laboratory at December 31, 1989, and its support, revenues, expenses and changes in fund balances for the year then ended in conformity with generally accepted accounting principles.

As disclosed in Note L to the financial statements, the Laboratory changed its method of accounting for current restricted funds in 1989.

Our audit was conducted for the purpose of forming an opinion on the basic financial statements taken as a whole. The supplemental schedules of support, revenues, expense and changes in fund balances for current funds (Schedule I), endowment funds (Schedule II) and plant funds (Schedule III) as of December 31, 1989, are presented for purposes of additional analysis and are not a required part of the basic financial statements. Such information has been subjected to the auditing procedures applied in the audit of the basic financial statements and, in our opinion, is fairly stated, in all material respects, in relation to the basic financial statements taken as a whole.

Coopers & Lybrand

Boston, Massachusetts
April 20, 1990

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1989
(with comparative totals for 1988)

ASSETS	*1989*	*1988*
Cash and savings deposits	$ 496,014	$ 199,970
Money market securities (Notes B and H)	965,000	1,750,000
Accounts receivable, net of allowance for uncollectible accounts	416,744	576,355
Receivables due for costs incurred on grants and contracts	1,200,558	836,608
Other assets	35,098	50,370
Total current assets	3,113,414	3,413,303
Investments, at market (Notes B and H)	15,971,459	11,401,121
Deposits with trustees (Note G)	133,000	—
Land, buildings and equipment (Notes B and C)	20,869,811	19,682,987
Less accumulated depreciation	(8,858,047)	(8,268,927)
	12,011,764	11,414,060
Total assets	$31,229,637	$26,228,484

The accompanying notes are an integral part of the financial statements.

LIABILITIES AND FUND BALANCES	*1989*	*1988*
Current portion of long-term debt	$ 65,000	$ 49,968
Accounts payable and accrued expenses	1,044,044	510,559
Deferred income	120,137	116,345
Total current liabilities	1,229,181	676,872
Mortgage and notes payable (Note G)	1,265,000	1,155,489
Deferred support (Note L)	4,450,222	2,951,662
Annuities payable (Note B)	105,355	—
Total liabilities	7,049,758	4,784,023
Current unrestricted fund balances	21,030	13,275
Endowment funds:		
Quasi-endowment unrestricted	426,982	409,997
Endowment, income for restricted purposes	4,804,006	3,595,974
Quasi-endowment restricted	4,480,887	3,913,255
Endowment, income for unrestricted purposes	3,234,878	2,850,409
	12,519,771	10,359,638
	12,946,753	10,769,635
Plant fund balances:		
Unrestricted	10,272,954	10,251,388
Restricted	790,322	29,922
Repairs and replacement reserve	148,820	380,241
	11,212,096	10,661,551
Total liabilities and fund balances	$31,229,637	$26,228,484

MARINE BIOLOGICAL LABORATORY

STATEMENT OF SUPPORT, REVENUES, EXPENSES AND CHANGES IN FUND BALANCES

for the year ended December 31, 1989
(with comparative totals for 1988)

	Current Funds		Endowment Funds		Plant Funds		1989 Total All Funds	1988 Total All Funds
	Unrestricted	Restricted	Unrestricted	Restricted	Unrestricted	Restricted		
SUPPORT AND REVENUES:								
Grant reimbursement of direct cost		$5,245,497				$574,821	$ 5,820,318	$4,117,945
Recovery of indirect costs related to research and instruction programs	$2,948,406						2,948,406	2,507,859
Tuition		440,562					440,562	399,888
Support activities:								
Dormitories	869,768						869,768	883,697
Dining hall	632,463						632,463	674,232
Library	249,128						249,128	209,134
Biological Bulletin	188,681						188,681	180,511
Research services	494,287						494,287	548,353
Marine resources	141,143						141,143	160,857
Investment income	427,272	499,929				15,750	942,951	675,268
	5,951,148	6,185,988				590,571	12,727,707	10,357,744
Gifts (Note I)	875,985	2,972,609	$ 38	$ 747,387		1,572	4,597,591	3,175,901
Change in deferred support	—	(1,498,560)	—	—		—	(1,498,560)	(1,298,328)
	875,985	1,474,049	38	747,387		1,572	3,099,031	1,877,573
Miscellaneous revenue	104,743	223,615					328,358	343,379
Total support and revenues	6,931,876	7,883,652	38	747,387		592,143	16,155,096	12,578,696

EXPENSES:								
Instruction		1,328,404					1,328,404	1,293,092
Research		5,070,730					5,070,730	3,931,936
Scholarships and stipends		233,511					233,511	187,022
Support activities:								
Dormitories	736,272						736,272	632,666
Dining hall	543,797						543,797	590,782
Library	606,691	154,963					761,654	564,104
Biological Bulletin	192,741						192,741	165,248
Research services	615,146	228,365					843,511	657,542
Marine resources	373,571						373,571	386,358
Administration	1,897,994						1,897,994	1,492,294
Sponsored projects administration	316,501						316,501	266,389
Plant operations	1,481,382						1,481,382	1,243,015
Depreciation					$ 589,120		589,120	561,239
Other	38,460	526,936			2,502	37,986	605,884	677,525
Total expenses	6,802,555	7,542,909	—	—	591,622	37,986	14,975,072	12,649,212
Excess (deficit) of support and revenues over expenses	129,321	340,743	38	747,387	(591,622)	554,157	1,180,024	(70,516)
Realized gains on investments			896	362,459	197,498		560,853	292,913
Unrealized gains on investment		30,672	3,942	959,927			994,541	231,996
Total gain on investments	—	30,672	4,838	1,322,386	197,498	—	1,555,394	524,909
Transfers	(121,566)	(371,415)	12,109	(90,360)	415,690	(25,178)	—	—
Net change in fund balances	7,755	—	16,985	2,160,133	21,566	528,979	2,735,418	454,393
Fund balances, beginning of year, as previously reported								22,643,402
Cumulative effect of change in accounting for current restricted funds (Note L)								(1,653,334)
Fund balances, beginning of year	13,275	—	409,997	10,359,638	10,251,388	410,163	21,444,461	20,990,068
Fund balances, end of year	$ 21,030	—	$426,982	$12,519,771	$10,272,954	$939,142	$24,179,879	$21,444,461

The accompanying notes are an integral part of the financial statements.

Marine Biological Laboratory

Notes to Financial Statements

A. *Purpose of the Laboratory:*

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

B. *Significant accounting policies:*

Basis of presentation—fund accounting

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of fund accounting. This is the procedure by which resources are classified into separate funds in accordance with specified activities or objectives. Separate accounts are maintained for each fund; however, in the accompanying financial statements, funds that have similar characteristics have been combined into fund groups. Accordingly, all financial transactions have been recorded and reported by fund group.

Externally restricted funds may only be utilized in accordance with the purposes established by the donor or grantor of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue as the related costs are incurred (see Note L).

Endowment funds are subject to restrictions requiring that the principal be invested in perpetuity with income available for use for restricted or unrestricted purposes by the Laboratory. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds; however, the principal of these funds may be expended for various restricted and unrestricted purposes.

Fixed assets

Fixed assets are recorded at cost. Depreciation is computed using the straight-line method over estimated useful lives of fixed assets.

Contracts and grants

Revenues associated with contracts and grants are recognized in the statement of support, revenues, expenses and changes in fund balances as the related costs are incurred (see Note L). The Laboratory reimbursement of indirect costs relating to government contracts and grants is based on negotiated indirect cost rates with adjustments for actual indirect costs in future years. Any over or underrecovery of indirect costs is recognized through future adjustments of indirect cost rates.

Investments

Investments purchased by the Laboratory are carried at market value. Money market securities are carried at cost, which approximates market value. Investments donated to the Laboratory are carried at fair market value at the date of the gift. For determination of gain or loss upon disposal of investments, cost is determined based on the average cost method.

The Laboratory is the beneficiary of certain endowment investments, reported in the financial statements, which are held in trust by others. Every ten years the Laboratory's status as beneficiary of these funds is reviewed to determine that the Laboratory's use of these funds is in accordance with the intent of the funds. The market values of these investments are $4,039,803 and $3,551,482 at December 31, 1989, and 1988, respectively.

Investment income and distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the market value unit basis (Note M).

Annuities payable

Amounts due to donors in connection with gift annuities is determined based on remainder value calculations which generally assure a rate of return at 10%, maximum payout terms of nineteen years, and interest payout rate of 8%.

C. *Land, buildings, and equipment:*

The following is a summary of the unrestricted plant fund assets:

	1989	*1988*
Land	$ 840,594	$ 689,660
Buildings	16,926,715	16,694,233
Equipment	2,521,904	2,299,094
Construction in progress	580,598	—
	20,869,811	19,682,987
Less accumulated depreciation	(8,858,047)	(8,268,927)
	$12,011,764	$11,414,060

D. *Retirement fund:*

On May 23, 1989, the Laboratory terminated its noncontributory defined benefit pension plan, which covered substantially all employees. Benefits earned by employees under the terminated plan became fully vested and were distributed to plan participants.

Net pension cost for fiscal year ending December 31, 1989 was:

Service cost	$ 27,041
Interest cost	173,227
Actual return on plan assets	(296,846)
Net amortizations and deferrals	82,291
Net periodic pension income	$ (14,287)

On February 17, 1989, in anticipation of the plan termination, effective May 23, 1989, the Laboratory froze future benefit accruals. The accumulated benefit obligation of $2,569,454 was settled through the purchase of non-participating annuity contracts and distribution of lump sum settlements. Because all excess assets were allocated among the participants, the Laboratory recognized no curtailment gain or loss. There was a settlement gain of $289,650 attributable to amounts previously accrued by the Laboratory and the plan ceased to exist as an entity.

A portion of the settlement gain of $192,285 was used to account for separation agreements with certain current employees. The balance of the settlement gain, $97,365 was returned to grants and contracts.

The Laboratory participates in the defined contribution pension program of the Teachers Insurance and Annuity Association. Expenses amounted to $393,422 in 1989 and $130,677 in 1988.

E. *Restricted pledges and grants:*

As of December 31, 1989, the Laboratory reported active pledge and grant commitments outstanding of $1,073,986 (unaudited) to be received. The restricted pledges are not included in the financial statements since it is not practicable to estimate the net realizable value of such pledges. Restricted pledges of $978,786 and $95,200 are scheduled to be paid in 1990 and 1991, respectively.

F. *Interfund borrowings:*

Current fund interfund balances at December 31 are as follows:

	1989	*1988*
Due to restricted endowment fund	$(2,190)	$(31,600)
Due to restricted quasi-endowment funds	(200)	—
	$(2,390)	$(31,600)

G. *Mortgage and notes payable:*

Long-term debt at December 31, 1989 amounted to $1,330,000. The aggregate amount of redemption due for each of the next five fiscal years is as follows:

	Amount
1990	$ 65,000
1991	65,000
1992	60,000
1993	60,000
1994	60,000
Thereafter	1,020,000
	1,330,000
Less current portion	65,000
	$1,265,000

During 1989, the Laboratory issued $1,330,000 Massachusetts Industrial Finance Authority (MIFA) Series 1989 Bonds, which pay varying annual interest rates and mature on October 31, 2011.

The bonds are payable annually with the first payment of $65,000 due October 1, 1990. The interest rate is adjustable and was 6.5% at December 31, 1989. In compliance with the Laboratory's MIFA bond indenture, deposits with Shawmut Bank, as trustee, represent investments in the debt service reserve fund of $133,000.

The Series 1989 bonds are collateralized by a first mortgage on certain Laboratory property.

H. *Investments:*

The following is a summary of the cost and market value of investments at December 31, 1989 and 1988 and the related investment income and distribution of investment income for the years ended December 31, 1989 and 1988.

	Cost		*Market*		*Investment Income*	
	1989	*1988*	*1989*	*1988*	*1989*	*1988*
Endowment and quasi-endowment						
U.S. Government securities	$ 2,595,407	$ 1,328,927	$ 2,607,537	$ 1,323,105	$134,394	$ 95,234
Corporate fixed income	5,900,736	3,124,493	6,032,642	3,131,404	363,439	257,692
Common stocks	3,392,001	3,717,850	5,901,724	5,375,980	196,452	211,319
Preferred stock	—	—	—	—	—	250
Money market securities	595,467	916,280	593,544	916,280	87,994	49,995
Real estate	345,749	15,749	345,749	15,749	—	—
Total	12,829,360	9,103,299	15,481,196	10,762,518	782,279	614,490
Less custodian and management fees					(49,318)	(44,073)
Total					732,961	570,417
Restricted current fund						
Certificates of deposits	490,263	638,603	490,263	638,603	34,785	38,603
Money market securities	965,000	1,750,000	965,000	1,750,000	175,205	66,248
Total	1,455,263	2,388,603	1,455,263	2,388,603	209,990	104,851
Total investments	$14,284,623	$11,491,902	$16,936,459	$13,151,121	$942,951	$675,268

I. *Gift support for instruction:*

Unrestricted gifts includes $406,524 of gifts for the support of the Laboratory's instruction program available for indirect costs attributable to the instruction program.

J. *Litigation:*

The Laboratory is involved in litigation on several matters and is subject to the possibility of certain claims arising in the normal course of business, none of which, in the opinion of management are expected to have a materially adverse effect on the Laboratory's financial position.

K. *Tax-exempt status:*

The Laboratory is exempt from federal income tax under Section 501(c)3 of the Internal Revenue Code.

L. *Change in accounting method for current restricted funds*

Effective January 1, 1989, the Laboratory adopted the accounting policy of deferring recognition of revenue on current restricted funds until the related costs are incurred. Amounts received in excess of expenses are recorded as deferred support. This change has been retroactively applied to the fund balances as of January 1, 1988. The cumulative decrease in the fund balances was $4,450,222 and $2,951,662 at December 31, 1989 and 1988, respectively. The following summarizes the activity in the deferred support account for 1989 and 1988, respectively:

	1989	*1988*
Balance at beginning of year	$2,951,662	$1,653,334
Additions:		
Gifts, endowment income and grants received	9,382,212	7,298,318
Unrealized gains	30,672	—
Deductions:		
Funds expended under gifts and grants	7,542,909	6,047,936
Transfers	371,415	(47,946)
Balance at end of year	$4,450,222	$2,951,662

M. *Accounting for pooled investments:*

The major portion of investment assets is pooled for investment purposes with each participating fund subscribing to, or disposing of, units at market value at the beginning of the current quarter. The unit participation of the funds at December 31, 1989 is as follows:

	1989
Endowment and similar funds:	
Quasi-unrestricted	3,975
Quasi-restricted	7,551
Restricted endowment	37,220
	48,746

Pooled investment activity on a per-unit basis was as follows:

Unit value at beginning of year	$100.00
Unit value at end of year	107.42
Increase in realized and unrealized appreciation	7.42
Net income earned on pooled investments	5.61
Total return on pooled investments	$ 13.03

Investment income is distributed to individual funds as earned.

SCHEDULE I

MARINE BIOLOGICAL LABORATORY

STATEMENT OF SUPPORT, REVENUES, EXPENSES AND CHANGES IN FUND BALANCES

CURRENT FUNDS

for the year ended December 31, 1989

	Operating Fund	*Housing Enterprises Fund*	*Total Current Unrestricted Fund*	*Current Restricted Fund*	*Total*
SUPPORT AND REVENUES:					
Grant reimbursement of direct costs				$5,245,497	$ 5,245,497
Change in deferred support					
Recovery of indirect costs related to research and instruction programs	$2,948,406		$2,948,406		2,948,406
Tuition				440,562	440,562
Support activities:					
Dormitories		$869,768	869,768		869,768
Dining hall	632,463		632,463		632,463
Library	249,128		249,128		249,128
Biological Bulletin	188,681		188,681		188,681
Research services	494,287		494,287		494,287
Marine resources	141,143		141,143		141,143
Investment income	427,272		427,272	499,929	927,201
	5,951,148		5,951,148	6,185,988	12,137,136
Gifts	875,985		875,985	2,972,609	3,848,594
Change in deferred support	—		—	(1,498,560)	(1,498,560)
	875,985		875,985	1,474,049	2,350,034
Miscellaneous revenue	104,743		104,743	223,615	328,358
Total support and revenues	6,062,108	869,768	6,931,876	7,883,652	14,815,528
EXPENSES:					
Instruction				1,328,404	1,328,404
Research				5,070,730	5,070,730
Scholarships and stipends				233,511	233,511
Support activities:					
Dormitories		736,272	736,272		736,272
Dining hall	543,797		543,797		543,797
Library	606,691		606,691	154,963	761,654
Biological Bulletin	192,741		192,741		192,741
Research services	615,146		615,146	228,365	843,511
Marine resources	373,571		373,571		373,571
Administration	1,843,190	54,804	1,897,994		1,897,994
Sponsored projects administration	316,501		316,501		316,501
Plant operations	1,481,382		1,481,382		1,481,382
Other	38,460		38,460	526,936	565,396
Total expenses	6,011,479	791,076	6,802,555	7,542,909	14,345,464
Excess (deficit) of support and revenues over expenses	50,629	78,692	129,321	340,743	470,064
Unrealized gain on investments				30,672	30,672
TRANSFERS AMONG FUNDS:					
Debt service	(5,225)	(29,548)	(34,773)		(34,773)
Acquisition of fixed assets				(335,836)	(335,836)
Net transfer to restricted plant fund				31,743	31,743
Transfers to unrestricted plant fund	(2,502)		(2,502)		(2,502)
Housing transfer		(49,144)	(49,144)		(49,144)
Endowment transfers				(3,774)	(3,774)
Instruction transfer	(7,160)		(7,160)	7,160	—
Capitalize ecosystems income				(90,822)	(90,822)
Other transfers	(27,987)		(27,987)	20,114	(7,873)
Total transfers among funds	(42,874)	(78,692)	(121,566)	(371,415)	(492,981)
Net change in fund balances	7,755	—	7,755	—	7,755
Fund balances, beginning of year	13,275	—	13,275	—	13,275
Fund balances, end of year	$ 21,030	—	$ 21,030	—	$ 21,030

SCHEDULE II

MARINE BIOLOGICAL LABORATORY

STATEMENT OF SUPPORT, REVENUES, EXPENSES AND CHANGES IN FUND BALANCES

ENDOWMENT FUNDS

for the year ended December 31, 1989

	Unrestricted	*Restricted*			
	Quasi-endowment	*Endowment, income for unrestricted purposes*	*Endowment, income for restricted purposes*	*Quasi-endowment*	*Total*
SUPPORT AND REVENUES:					
Gifts	$ 38		$ 740,427	6,960	$ 747,425
Total support and revenues	38		740,427	6,960	747,425
Excess of support and revenues over expenses	38		740,427	6,960	747,425
Realized gain on investments	896	$ 98,242	112,701	151,516	363,355
Unrealized gains on investments	3,942	286,227	354,904	318,796	963,869
Total gain on investments	4,838	384,469	467,605	470,312	1,327,224
TRANSFERS AMONG FUNDS:					
Capitalize ecosystems income				90,822	90,822
Endowment transfers				3,774	3,774
Other transfers	12,109			(4,236)	7,873
Total transfers among funds	12,109	—	—	90,360	102,469
Net change in fund balances	16,985	384,469	1,208,032	567,632	2,177,118
Fund balances, beginning of year	409,997	2,850,409	3,595,974	3,913,255	10,769,635
Fund balances, end of year	$426,982	$3,234,878	$4,804,006	$4,480,887	$12,946,753

Schedule III

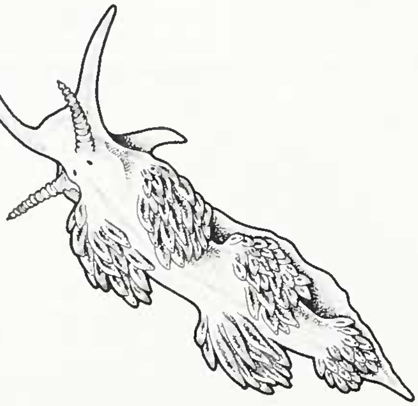
MARINE BIOLOGICAL LABORATORY

Statement of Support, Revenues, Expenses and Changes in Fund Balances

Plant Funds

for the year ended December 31, 1989

	Unrestricted	*Restricted*	*Repairs and Replacement Reserve*	*Total*
SUPPORT AND REVENUES:				
Grant reimbursement of direct costs		$574,821		$ 574,821
Investment income		15,750		15,750
Gifts		1,572		1,572
Total support and revenues		592,143		592,143
EXPENSES:				
Depreciation	$ 589,120			589,120
Other	2,502		$ 37,986	40,488
Total expenses	591,622	—	37,986	629,608
Excess (deficit) of support and revenues over expenses	(591,622)	592,143	(37,986)	(37,465)
Realized gains on investments	197,498	—	—	197,498
TRANSFERS AMONG FUNDS:				
Debt service	34,773			34,773
Acquisition of fixed assets	578,415		(242,579)	335,836
Net transfer to restricted plant fund	(200,000)	168,257		(31,743)
Transfers to unrestricted plant fund	2,502			2,502
Housing transfers			49,144	49,144
Total transfers among funds	415,690	168,257	(193,435)	390,512
Net change in fund balances	21,566	760,400	(231,421)	550,545
Fund balances, beginning of year	10,251,388	29,922	380,241	10,661,551
Fund balances, end of year	$10,272,954	$790,322	$148,820	$11,212,096



Report of the Librarian

During the last two years, the Library has changed tremendously. We've been entering the electronic age, and in our leap forward we inadvertently missed being included in the MBL's 1988 Annual Report. The record should show that the Library was not in a state of somnolence, but rather in a state of rapid growth, expansion, excitement, and innovative planning.

The MBL Centennial year

1988 was a year for experiencing the Centennial. The entire library staff was involved in organizing and volunteering for many exciting Centennial events. The Futures in Science Program, the Centennial Year Summer High School Science Teacher Award, the Symposium on Learning and Memory, the History of Science Lecture Series, and Old Timers' Day were given special emphasis by the Library. The latter event brought back many Library users from the lab's early days, and their visits gave us an opportunity to explore the Library's history with those who were a part of it. It was a joy to work with John (Stubby) and Julie Rankin, Donald Zinn, Sears Crowell, Garland Allen and John Valois in planning "Old Timers Day." As a tribute to Dr. Rankin, who died before the event he had worked so hard to bring about, many of his friends and former students have contributed to a book fund in his memory. The third floor of the Library now has a "Stubby" Rankin shelf for monographics on systematic ecology and invertebrate zoology.

In 1988, the Library received the Montgomery Collection—a valuable collection of books, journals, and diaries describing Arctic and Antarctic expeditions. The gift includes Captain James Cook's *A Voyage to the Pacific Ocean*—the record of his expedition to the South Pacific on the ships HMS RESOLUTION and HMS DISCOVERY during the years 1776 to 1780. The volume includes some first edition maps of the voyage. This valuable addition to the Library was the gift of the Hugh and Raymond Montgomery families, whose ties to the MBL go back to the lab's early years when Priscilla Montgomery was the first full-time librarian. Her husband, T. H. Montgomery, was a cytologist from the University of Pennsylvania and an MBL trustee. The books were collected by their nephew, Newcomb Thompson Montgomery, and are now cataloged and housed in the Library's Rare Books Room.

Also in 1988, Dr. and Mrs. Robert Huettner donated a collection of MBL photographs taken by Robert's father, Alfred Huettner, in the 1920s and '30s. An exhibit of the photos was prepared by Ruth Davis, Library Archivist, and held in the Meigs Room during the Centennial summer. Many of the photographs, which capture the life of MBL scientists and their families, are still on display in the Library and the Candle House.

Ruth Davis and Jane Maienschein, the co-director of the History of Science summer course, prepared a popular history of the MBL titled *One Hundred Years Exploring Life, 1888–1988*, published by Jones and Bartlett Publishers, Boston. It is a commemorative volume about the MBL's first one hundred years containing many previously unpublished photos that had been found in the MBL Archives. Ruth also oversaw the filming done in the Archives for Newton's Apple, a segment of the popular PBS series NOVA.

The small and large reading rooms underwent face lifts in preparation for the Centennial and for their dedication to two families who have been generous benefactors to the MBL. The Albert M. and Ellen R. Grass Reference Room was dedicated on July 15, 1988 and the Charles Ulbrick Bay Reading Room was dedicated on August 12, 1988. The Josephine and Charles Ulrick Bay Foundation gave a one million dollar gift to the Library to complete the 2.5 million Mellon challenge grant. At the dedication, Frederick Bay spoke of his father, Charles U. Bay:

> "In the foundation that bears his name, a small fragment of Charles U. Bay's creative industry remains, to provide what is called, in the fashion of our time, "seed money," which makes possible the germination of ideas deemed too risky or unworkable by tangled government or the ever short-sighted profit-conscious sector.
>
> Today, then, marks the placing of a store of seeds, if you will, here at the MBL, from which we trust a thousand creative flowerings will bloom."

We look forward to the challenge.

The next century

The Library has embarked on a path into the next century; we are modernizing and changing the way our Library gathers and disseminates information. We are building a network that will enable scientists, information producers, vendors, and our entire research community, to communicate with each other locally and world-wide. With this vision, the Howard Hughes Medical Institute awarded the Library one million dollars for planning and development. This award prompted the creation of the Office of Library Planning; Cathy Norton has been named Director. A Library Planning Committee, consisting of nationally recognized experts in science and information research, was subsequently appointed. The members of the committee are:

Dr. Edward A. Adelberg, Deputy Provost, Yale Medical School, Chairman of the Planning Committee
Dr. Garland E. Allen, Washington University
Dr. Carl O. Bowin, Senior Scientist, Geology and Geophysics Department, WHOI
Dr. John E. Dowling, Professor of Natural Sciences, Harvard University
Dr. Alan E. Erickson, Cabot Science Library, Harvard University
Dr. David Glover, Research Associate, Chemistry Department, WHOI
Dr. Joel C. Goldman, Senior Scientist, Biology Department, WHOI
Richard Lucier, Director, Laboratory for Applied Research in Academic Information, The William H. Welch Medical Library, The Johns Hopkins University
Andrew R. Maffei, Research Specialist, Applied Ocean Physics and Engineering Department, WHOI
Nina W. Matheson, Director, The William H. Welch Medical Library, The Johns Hopkins University
Dr. Edward Rastetter, Assistant Scientist, Ecosystems Center, MBL
Dr. Temple Smith, Dana-Farber Cancer Institute
Dr. John Stegeman, Senior Scientist, Biology Department, WHOI

The library Planning Committee was charged with developing a long-range plan for the Library in terms of the systems and resources needed to support the research and educational programs of the entire Woods Hole scientific community in the Twenty-First Century. In August 1989, Chairman Adelberg reported to the Trustees the goals, objectives, and recommendations put forth by the committee. These clearly state that the Library must prepare for, and provide the scientific community with, state-of-the-art information technology and services, as well as assistance and training in the use of the new technologies.

The MBL/WHOI Office of Library Planning, with the advise and support of the Library Planning Committee, has begun to establish a network that will bring the Library to the laboratory bench and take the Library and its users out into a vast national computer network. With MBLnet and the On-line Scientific Information System (OSIS), developed in the library this year, it will be possible to access databases on CD-ROMs and disks, use electronic-services, build databases, and transform the way we manage information at the MBL.

Our scientists need information that is being delivered and packaged in electronic form, and we must be able to manipulate this data by being sufficiently computer literate to use it. The Library has always been the liaison between information and those who need it; with this in mind, the Library staff has dedicated themselves to becoming familiar with all formats of information in today's myriad of publication modes and with the methods of delivering this information.

New technical positions were added, and some staff members were reassigned during 1989 to accomplish the goals and objectives established by the Library Planning Committee. The leadership of the Library has changed: the head librarian, Jane Fessenden, moved to the newly created position of Special Collections Librarian in the Rare Books area, and Cathy Norton was appointed as Acting Librarian. A national search is underway for a Director. Three new staff positions—network technical advisor, computer users technician, and secretary to the Director of Library Planning—were added under the Hughes grant to help support the network and the move towards an "electronic library."

During the summer of 1989, a pilot project was initiated to test new methods of accessing scientific information. The broadband connection from the Woods Hole Oceanographic Institution was installed in the Library and in a teaching lab in Loeb. This connection allowed the Library to deliver various tools and services to researchers who were connected to the network and also allowed the Library to offer e-mail services to the MBL community. A number of databases were loaded on PCs, and access to CD-ROM data files was made available to Library users. A prototype information retrieval system was installed in the Library as a beta test site for the American Society for Microbiology (ASM). All journals published in the past three years by ASM are retrievable on this system with full text and graphics. The response to and acceptance of these new tools by Library users was overwhelmingly positive. The initiative that began with the Hughes award has led to the establishment of numerous effective partnerships and active participation of scientists and information professionals. These interactions will enhance research productivity and educational goals in the Woods Hole scientific community.

Other initiatives

The National Science Foundation Summer Research Experience for Young Scholars was conceived and received funding this year through the efforts of the Office of Library Planning and Dr. Jay Tashiro of Simon's Rock of Bard College. The program, which will continue each summer, allows tenth and eleventh grade students to explore new ideas in science and technology in a four-week "hands-on" research experience. Students begin the program at Simon's Rock College and then come to Woods Hole for the research and library component of their projects. These new initiatives have broaden the scope of the Library's user population and will help bring stability and cost-support structure to the Library.

The Library joined with eighteen public libraries on the Cape and with Cape Cod Community College to form CLAMS—Cape Library Automated Material Sharing. Funding provided to CLAMS by the Massachusetts Board of Library Commissioners is supporting our efforts to automate the MBL/WHOI Library's catalog and circulation systems as part of a Cape-wide system. The system is up and the librarians are in training on the system; bar-coding of our book collection will be completed in 1990.

The International Association of Marine Sciences Libraries and Information Centers (IAMSLIC) held their Annual Meeting this year in Bermuda. Both Cathy Norton and Judy Ashmore gave papers on fund raising in libraries and the use of library services in relation to these activities.

This report has stressed the future of the library, but I cannnot close without a glance at the past. In 1979, when Jane Fessenden had been the Librarian for 17 years, she wrote a report for the Library Committee, which included the following lists of goals for the ten years to come:

1. To offer instruction in the use of bibliographic services.
2. To increase the book budget for greater benefit to students and additional reference material.
3. To add new systems for more rapid delivery of xeroxed articles.
4. To computerize our serials list.
5. Computerize circulation records and sign-out procedures.
6. To add new bibliographic search services.
7. Bind more of our reprint collection.
8. To hire robots to shelve, and reshelve, and re-shelve . . .

At the end of 1989 we see that these tasks (alas not #8) have been accomplished and continue to be worthy pursuits. This year Ms. Fessenden has become the Special Collections Librarian in the Rare Books Room. The library staff and her many friends at the Laboratory have been the beneficiaries of Jane's library stewardship, and we look forward to her endeavors to bring order to the history of MBL—a Herculean task.

As one may be able to ascertain from this report, the seeds that were planted by the Andrew W. Mellon Foundation, the Bay Foundation, and the Howard Hughes Medical Institute fell on fertile ground. Undoubtedly their blossoms will flourish with the continued nurturing of our unique scientific community.

—Catherine Norton

G Liles

Educational Programs

Summer Courses

Biology of Parasitism (June 11 to August 11)

Directors

John E. Donelson, University of Iowa College of Medicine
Carole Long, Hahnemann Medical College

Faculty

Steven Anderson, University of Cincinnati
Steve Beverley, Harvard Medical School
Ted Bianco, Imperial College of Science and Technology, UK
Bruce M. Christensen, University of Wisconsin
Patrick Farley, Hahnemann Medical College
Brian Finnegan, University of Pennsylvania
Steven L. Hajduk, University of Alabama, Birmingham
Michael Harris, University of Alabama, Birmingham
Mary Hartman, University of Kentucky
Peter Kima, Hahnemann Medical College
James Lok, University of Pennsylvania
Rick Martin, Boehringer Mannheim Corp.
David Moser, University of Iowa
David Russell, New York University
David Sachs, NIH/NIAID
Larry Simpson, University of California, Los Angeles
Sam Turco, University of Kentucky Medical School
Mervyn J. Turner, Merck, Sharp & Dohme Laboratories
Wenlin Zeng, University of Iowa

Lecturers (in order of appearance)

Irwin Sherman, University of California, Riverside
Paul Englund, Johns Hopkins School of Medicine
Buddy Ullman, University of Oregon Health Science Center
Alan Sher, NIH/NIAID
Ruth Nessenzweig, NYU Medical Center
Victor Nussenzweig, NYU Medical Center
Elmer Pfefferkorn, Dartmouth Medical School
Paul Knopf, Brown University
Andrew Spielman, Harvard School of Public Health
John Mansfield, University of Wisconsin
Anthony Cerami, Rockefeller University
Jim McKerrow, University of California, San Francisco
Vaughn Kirchhoff, University of Iowa
Barry Bloom, Albert Einstein College of Medicine
Jim Kazura, Case Western Reserve University
Tim Nilson, Case Western Reserve University
Jack Strominger, Harvard University
Dyann Wirth, Harvard School of Public Health
Richard Young, Whitehead Institute, MIT
Debbie Peattie, Harvard School of Public Health
Hugh Taylor, Johns Hopkins University
Hank Seifert, Northwestern University
Jeffrey Chulay, Walter Reed Army Institute of Research
George Nelson, Liverpool, England, UK
Jonathan Ravdin, University of Virginia

Students

Tamara Aboagye-Kwarteng, London School of Hygiene & Tropical Medicine, UK
Paulo Andrade, Federal University of Pernambuco, Brazil
Brenda Beerntsen, University of Wisconsin
Vladimir Correado, New York University
Philip Effron, Johns Hopkins University Medical School
Najib El-Sayed, Yale University

Phyllis Freeman-Junior, Meharry Medical College
Mary Gonzatti, University Simon Bolivar, Venezuela
Christopher Karp, Georgetown University
Marika Kullberg, University of Stockholm, Sweden
Lynn Morris, University of Oxford, UK
Dania Richter, Freie University Berlin, FRG
David Siegel, University of Wisconsin
Walter Weiss, Naval Medical Research Institute
David Williams, University of Illinois
Lilian Yepez-Mulia, CINVESTAV-IPN, Mexico

Embryology (June 18 to July 29)

Director
Eric H. Davidson, California Institute of Technology

Faculty and Staff
Raffi Aroian, California Institute of Technology
Carol Burdsal, Duke University
Emmeline Chiao, Bryn Mawr College
Douglas DeSimone, University of Virginia
Charles Ettensohn, University of Pittsburgh
Richard A. Firtel, University of California, San Diego
Joseph Gall, Carnegie Institution of Washington
Susan Halsell, California Institute of Technology
Janet Heasman, University of Cambridge, UK
Peter Howard, University of California, San Diego
Linda Huffer, Marine Biological Laboratory
Andrew D. Johnson, University of California, Irvine
Ross Kinloch, Roche Institute of Molecular Biology
David Kirk, Washington University
Mary LaGrange, University of Massachusetts, Amherst
Michael Levine, Columbia University
Howard Lipshitz, California Institute of Technology
David McClay, Duke University
Doug Melton, Harvard University
Steve McKnight, Carnegie Institution of Washington
Robert W. Nickells, California Institute of Technology
Jane Rigg, California Institute of Technology
Kenneth Robinson, Purdue University
Joan Ruderman, Harvard University
L. Dennis Smith, University of California, Irvine
Paul W. Sternberg, California Institute of Technology
Nicholas Torpey, University of Cambridge, UK
Raul Warrior, Columbia University
Paul M. Wassarman, Roche Institute of Molecular Biology
Richard Whittaker, Marine Biological Laboratory
Christopher Wylie, University of Cambridge, UK

Students
Mayi Arcellana-Panlilio, University of Calgary, Canada
Juliane Bernholz, University of Basel, Switzerland
Annette Boman, Johns Hopkins University
David Bumcrot, University of Pennsylvania
John Cunniff, University of Florida
Bernard Degnan, University of Queensland, Australia
Miguel Estevez, University of Missouri
Robert Goldstein, University of Texas, Austin
Anne Groell, University of California, Irvine
Theodor Haerry, University of Basel, Switzerland
Karen Jaques, University of Cambridge, UK
Mark Johnson, Case Western Reserve University
Robert Kelsh, University of Cambridge, UK
Min Ku, Harvard University
Katherine Ladner, University of California, Irvine
Chengyu Liu, University of California, Irvine
Adnan Nasir, University of Rochester
Haroki Nishida, Kobe University, Japan
Franco Palla, Universita di Palermo, Italy
David Ranson, University of Virginia
Patrick Schnegelsberg, Clark University
Ekkehard Schulze, University of Goettingen, FRG
Robert Sparks, North Dakota State University
Dmitry Tchurikov, Academy of Science, USSR
Andre Van Loon, State University of Utrecht, The Netherlands
Menquing Xiang, University of Texas

Marine Ecology (June 18 to July 29)

Director
J. Woodland Hastings, Harvard University

Faculty
Hans Paerl, University of North Carolina, Chapel Hill
Dennis A. Powers, Hopkins Marine Station
Thomas T. Chen, University of Maryland
Carolyn A. Currin, University of North Carolina, Chapel Hill
Steven J. Giovannoni, Oregon State University
Julie Diane Kirshtein, University of North Carolina, Chapel Hill
Robert G. Rowan, Hopkins Marine Station
Bess B. Ward, University of California, Santa Cruz

Lecturers (in order of appearance)
Richard Ogden, Agouron Institute
Rick Carlton, Michigan State University
Brad Bebout, University of North Carolina, Chapel Hill

Lee Kerkhoff, Scripps Institution of Oceanography
John Stegeman, Woods Hole Oceanographic Institution
Beatrice Sweeney, University of California, Santa Barbara
Penny Chisholm, Massachusetts Institute of Technology
Colleen Cavanaugh, Harvard University
Katherine G. Field, Oregon State University
R. Olson, Woods Hole Oceanographic Institution
Ken Nealson, University of Wisconsin, Milwaukee

Students

Dror Angel, CUNY
Barbara Best, Columbia University
Daniel Brazeau, SUNY, Buffalo
Robert Browne, Wake Forest University
Mary-Alice Coffroth, SUNY, Buffalo
Hudson DeYoe, Bowling Green State University
Lynne Gilson, Harvard University
Alan Groeger, Murray State University
Matthew Hoch, University of Delaware
Jen-jen Lin, University of California, San Diego
Michael Montgomery, University of Colorado
Gisele Muller-Parker, University of Maryland
David Penny, University of Southern California
Carol Reeb, University of Georgia
Christopher Scholin, Woods Hole Oceanographic Institution
Steven Sczekan, North Carolina State University
Jeffrey Silberman, University of Miami
David Smith, University of California, San Diego
Peter Starkweather, University of Nevada
Tracy Stevens, Portland State University
John Stolz, University of Massachusetts
Tzung-horng Yang, University of California, San Diego

Microbiology *(June 11 to July 27)*

Directors

Ralph Wolfe, University of Illinois
E. Peter Greenberg, University of Iowa

Faculty

Frank Aeckersberg, Phillips-Universitat, Marburg, FRG
Richard M. Behmlander, University of Minnesota
Deborah Eastman, University of Minnesota
Kendall Gray, University of Southern California
Andrew M. Kropinsky, Queens University, Ontario, Canada
Carla Kuhner, University of Illinois
Scott G. Smith, University of Illinois
Friedrich Widdel, Phillips-Universitat, Marburg, FRG

Lecturers (in order of appearance)

Richard Blakemore, University of New Hampshire, Durham
Dennis Bazylinski, Woods Hole Oceanographic Institution
Richard Frankel, University of California, Santa Cruz
Barry Marrs, E. I. DuPont de Nemours & Co.
Douglas Youvan, Massachusetts Institute of Technology
Fevzi Daldal, University of Pennsylvania
Bert Ely, University of South Carolina
Linc Sonenshein, Tufts University
Wendy Champness, Michigan State University
David Gibson, University of Iowa
Caroline Harwood, University of Iowa
Nick Ornston, Yale University
Alan Hooper, University of Minnesota, St. Paul
Robert Tabita, Ohio State University
George Lorimer, E. I. DuPont de Nemours & Co.

Students

Alfredo Alder, Swiss Federal Institute, Switzerland
Porter Anderson, University of Rochester
Carol Arnosti, Woods Hole Oceanographic Institution
Simone Dannenberg, University of Konstanz, FRG
Joachim Ellermann, Philipps University, Marburg, FRG
Michael Ferris, Merck & Co.
Leslie Gregg, Yale University
Robert Gunsalus, University of California, Los Angeles
John Hawkins, University of Queensland, Australia
Min Kyung Kim, University of Iowa
Kathleen Ledyard, Massachusetts Institute of Technology/WHOI
Nicholas Mantis, Cornell University
Elaine Mirkin, University of Southern California
Esteban Monserrate, University of Massachusetts, Amherst
Flynn Picardal, University of Arizona
Bettina Rosner, University of Tübingen, FRG
Jan Rosnes, University of Bergen, Norway
Sigrid Schenk, Philipps University, Marburg, FRG
Imke Schroeder, University of California, Los Angeles
Hansruedi Siegrist, Swiss Federal Institute of Technology, Switzerland
Claudia Walter, University of Osnabruck, FRG

Neural Systems and Behavior *(June 11 to July 29)*

Director
Thomas Carew, Yale University

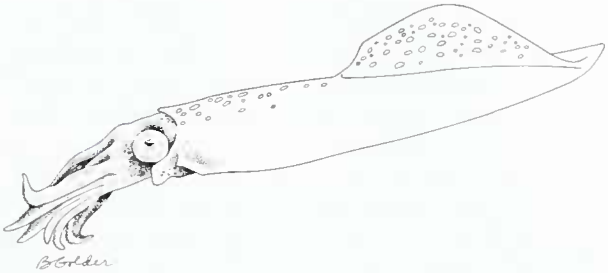
Faculty
James D. Angstadt, Emory University
Patrick Bateson, University of Cambridge, UK
Robert Barlow, Syracuse University
Alexander Borst, Tubingen, FRG
John Byrne, University of Texas Medical School
Ronald Calabrese, Emory University
Leonard Cleary, University of Texas Medical School
Hollis Cline, Stanford University
Martha Constantine-Paton, Yale University
Brian W. Edmonds, Columbia University
Ellen Elliott, Duke University Medical School
Russell D. Fernald, University of Oregon
Thomas M. Fischer, University of California, Riverside
Cole Gilbert, Indiana University
Dennis L. Gorlick, Columbia University
Sally G. Hoskins, CUNY
Gwen Jacobs, University of California, Berkeley
Darcy B. Kelley, Columbia University
Karla Kent, University of Arizona
Margarethe Kirchbaumer, City College of New York
John Koester, Research Center for Mental Hygiene
Masakazu Konishi, California Institute of Technology
Richard Levine, University of Arizona
Eduardo Macagno, Columbia University
Emilie Marcus, Yale University
Melanie Marin, Columbia University
Naomi Nagaya, University of Southern California
Michael Nusbaum, San Francisco State University
Edwin Rubel, University of Washington
Carla Shatz, Stanford University Medical School
Antonia Stephen, Brown University
Tim Tully, Brandeis University
Janis C. Weeks, University of Oregon
William Wright, Yale University

Students
Andriana Alcantara, University of Illinois
Viviana Berthoud, Albert Einstein College of Medicine
David Capco, Arizona State University
Robin Cloues, Harvard University
Richard Fay, Loyola University
Kent Fitzgerald, Yale University
Linda Goldstein, Indiana University
Keri Halsema, University of Southern California
Aaron Joseph, University of Washington
Kathleen Killian, Rutgers University
Sabine Kreissl, Freie Universitat, Berlin, FRG
Robert Lee, University of Colorado
Michael Nitabach, Massachusetts Institute of Technology
Joseph Pieroni, University of Texas Medical School
Joachim Schmidt, University of Constance, FRG
Philip Stoddard, University of Washington
Susan Swithers, Duke University
Dawn Tamarkin, University of Arizona
Robert Waldeck, Temple University
Debbie Wood, Georgia State University
Alex Chernajavsky, Yale University
Guylaine Durand, Albert Einstein College of Medicine
Bernhard Flucher, NIH/NINDS
Michael Grant, Case Western Reserve University
Hebe Guardioloa-Diaz, University of Michigan
Ekkehard Kasper, University of Oxford, UK
Kelley Kruger, Columbia University
Anne Metcalf, Yale University
Vincent O'Connor, University College, London, UK
Ning Qian, Johns Hopkins University
Linda Robertson, Northwestern University
David Stauffer, California Institute of Technology
David Welsh, Harvard Medical School

Neurobiology *(June 11 to August 19)*

Director
Arthur Karlin, Columbia University

Faculty
Brian Andrews, NIH/NINDS
Cynthia Czajkowski, Columbia University College of Physicians & Surgeons
Jan Paul De Weer, Duke University
Gerald Fischbach, Washington University School of Medicine
Robert French, University of Calgary, Canada
Sarah Garber, Stanford University
Linda M. Hall, Albert Einstein College of Medicine
James Huettner, Harvard Medical School
Laurinda A. Jaffe, University of Connecticut Health Center
Lily Jan, Howard Hughes Medical Institute
Yuh Nung Jan, Howard Hughes Medical Institute
Robert S. Kass, University of Rochester School of Medicine
Richard Kramer, Columbia University College of Physicians & Surgeons
Dennis Landis, Case Western Reserve University
Story Landis, Case Western Reserve University



Dennis Liu, Institute of Neuroscience
Craig C. Malbon, SUNY, Stony Brook
Gail Mandel, Tufts University School of Medicine
Steven Matsumoto, University of Arizona College of Medicine
Andres Matus, University of Basel, Switzerland
James McCarter, Princeton University
Christopher Miller, Brandeis University
Robert Miller, Case Western Reserve University
Thomas Reese, NIH
Bert Sakmann, Max-Planck-Inst. fur Biophysicalische Chemie, FRG
Bruce Schnapp, Boston University
Thomas I. Segerson, New England Medical Center
Michael Sheetz, Washington University School of Medicine
Steven A. Siegelbaum, Columbia University College of Physicians & Surgeons
Carolyn L. Smith, NIH/NINDS
Stefano Vicini, Georgetown University Medical School
Hsien-Yu Wang, SUNY, Stony Brook
Monte Westerfield, University of Oregon
William F. Wonderlin, University of Calgary, Canada

Lecturers (in order of appearance)

S. Siegelbaum, Columbia University College of Physicians & Surgeons
C. Miller, Brandeis University
S. Jones, Case Western Reserve University
R. French, University of Calgary, Canada
B. Sakmann, Max-Planck-Inst. fur Biophysicalische Chemie, FRG
S. Vicini, Georgetown University Medical School
R. Kass, University of Rochester School of Medicine
Andrew Mattox, Marine Biological Laboratory
George Augustine, University of Southern California
M. V. L. Bennett, Albert Einstein College of Medicine
Craig Malbon, SUNY Health Science Center
J. Schwartz
J. Stock
E. Holtzman
W. Stuhmer
G. Mandel, Tufts University School of Medicine
Linda M. Hall, Albert Einstein College of Medicine
Lily Jan, Howard Hughes Medical Institute
Gerald Fischbach, Washington University School of Medicine
Yuh Nung Jan, Howard Hughes Medical Institute
S. Landis, Case Western Reserve University
R. Miller, Case Western Reserve University
Monte Westerfield, University of Oregon
J. Huettner, Harvard Medical School
Carolyn Smith, NIH/NINDS

Students

Alex Chernjavsky, Yale University
Guylaine Durand, Albert Einstein College of Medicine
Bernhard Flucher, NIH/NINDS
Michael Grant, Case Western Reserve University
Hebe Guardiola-Diaz, University of Michigan
Ekkehard Kasper, University of Oxford, UK
Kelley Kruger, Columbia University
Anne Metcalf, Yale University
Vincent O'Connor, University College, London, UK
Ning Qian, Johns Hopkins University
Linda Robertson, Northwestern University
David Stauffer, California Institute of Technology
David Welsh, Harvard Medical School

Physiology *(June 11 to July 22)*

Director

Thomas Pollard, Johns Hopkins Medical School

Faculty

William Balch, Scripps Clinic & Research Foundation
Kerry Bloom, University of North Carolina
Orielan Condeelis
Gia Harewood, University of Rochester
Robert Jensen, Johns Hopkins Medical School
Kenneth Johnson, Pennsylvania State University
Johnny E. Jones, University of North Carolina, Chapel Hill
Karen Magnus, Case Western Reserve University
Marianne Manchester, University of North Carolina, Chapel Hill
Mark Mooseker, Yale University
Mark Paradise, Colorado College
Sandra Schmid, Scripps Clinic & Research Foundation
John Sinard, Johns Hopkins Medical School
Murray Stewart, Medical Research Council, Cambridge, UK

Lewis Tilney, University of Pennsylvania
Katherine Wilson, Johns Hopkins Medical School
Elaine Y. Yeh, University of North Carolina

Lecturers (in order of appearance)
Peter Novak, Yale Medical School
Ari Helenius, Yale University
Jim Rothman, Princeton University
Paul Lazarow, Rockefeller University
Don Newmeyer, La Jolla, CA
Reid Gilmore, University of Massachusetts
Paul Russell, Scripps Institution of Oceanography
Rick Klausner, NIH
Jim Paulson, University of Wisconsin
Linda Hickey
A. Szent-Györgyi, Brandeis University
Hugh Huxley, Brandeis University
Richard Vallee, Worcester Foundation for Experimental Biology
Edward Salmon, University of North Carolina
Steve Harrison, Harvard University
Jim Haber, Brandeis University
Mike Snyder, Yale University
John Pringle, Michigan University

Students
Kenneth Armour, St. Andrews University, Scotland
Jenny Baverstock, Wolfson College, Oxford, UK
William Bement, Arizona State University
Margaret Berg, Cornell University Medical School
Carol Berkower, Johns Hopkins School of Medicine
Annette Boman, Johns Hopkins University
Andrew Bullen, University of Texas, Austin
Lisa D'Andrea, Rutgers University
Robert DeBoy, Johns Hopkins University
Quan-Yang Dun, University of California, San Francisco
Alan Fanning, Yale University
Leslie Fischer, Columbia University
Laura Georgi, University of Missouri
Kevin Gibson, University of Pittsburgh
Heather Harper, Purdue University
Philip Hausman, Northwestern University Medical School
Philip Hertzler, Bodega Marine Laboratory
Lee Janson, Carnegie-Mellon University
Thomas Keating, University of Pennsylvania
Claudia Landis, University of California, San Francisco
Yaron Levy, Brandeis University
Cheryl Nehme, Northwestern University
Glen Nuckolls, University of North Carolina
Janet Padgett, Johns Hopkins University
Promod Pratap, SUNY Health Science Center
Victor Saavedra-Alanis, Baylor College of Medicine
Ivelisse Sanches, Hunter College of SUNY
Monica Shiel, Boston University Medical School
Amy Spater, Stanford University
Andreas Stemmer, University of Basel, Switzerland
Barbara Stolz, University of Basel, Switzerland
Jeanine Ursitti, University of Maryland
Maria Virata, Northwestern University
Sandra Williams, East Tennessee State University
Nan Wu, Brown University
Samuel Yiu, Harvard Medical School

Short Courses

Cellular Neurobiology in the Leech
(August 3 to 23)

Directors
Ken Muller, University of Miami School of Medicine
John Nicholls, Biocenter, Basel, Switzerland

Faculty
Susanna Blackshaw, Glasgow University, UK
Edwardo Macagno, Columbia University
Brian W. Payton, Memorial University School of Medicine, Newfoundland, Canada
David Weisblat, University of California, Berkeley

Lecturers
Ron Calabrese, Emory University
Larry Cohen, Yale University Medical School
Otto Friesen, University of Virginia
William Kristan, University of California, San Diego
William Ross, New York Medical College
Marty Shankland, Harvard Medical School
Genther Stent, University of California, Berkeley

Students
Thomas Becker, University of Koln, FRG
Lucy Diaz-Miranda, University of Puerto Rico
Tamar Gallily, Hebrew University of Jerusalem, Israel
Lisa Sara Gascoigne, University of London, UK
Simone Grumbacher, Biozentrum, Basel, Switzerland
Masako Isokawa-Akesson, Brain Research Institute, UCLA
Nechama Lasser-Ross, New York Medical College
Theres Luthi, The Rockefeller University
Mark Q. Martindale, Harvard Medical School
David C. Merz, McGill University

Jacqueline Miodownik, University of Miami
Thomas K. Morrissey, University of Miami
Thomas F. Schilling, University of Oregon
Martin Stephan, Max-Planck-Institut, FRG

History of Biology: Neurobiology and Behavior (July 30 to August 12)

Directors
E. Garland Allen, Washington University
Richard W. Burkhardt, Jr., University of Illinois
Jane Maienschein, Arizona State University

Lecturers
Colin Beer, Rutgers University
Michael Bennett, Albert Einstein Medical College
Anne Harrington, Harvard University
Sharon E. Kingsland, The Johns Hopkins University
Edward Manier, University of Notre Dame

Students
Joyce V. Cadwallader, St. Mary-of-the-Woods College
Eileen Crist, Boston University
Eunice A. Cronin, Belmont Abbey College
Marcia D. Edwards, The Thatcher School
Daniel R. Goodman, Harvard College
Christiane Groeban, Stazione Zoologica–Anton Dohrn, Italy
Ann E. Kammer, Arizona State University
Ronald J. Overmann, National Science Foundation
Michael Barry Reiner, Salem College
Robert C. Richardson, University of Cincinnati
Clark T. Sawin, Boston Veterans'Administration Medical Center
Jonathan S. Schiffamn, Yale School of Medicine
John R. Shaver, University of Puerto Rico
Jan Butin Sloan, University of Kansas

Methods in Computational Neuroscience
(August 6 to September 2)

Directors
James M. Bower, California Institute of Technology
Christof Koch, California Institute of Technology

Faculty
Paul Adams, SUNY, Stony Brook
Edward Adelson, Massachusetts Institute of Technology
Daniel Alkon, NIH/NINDS
Richard Andersen, Massachusetts Institute of Technology
Clay Armstrong, University of Pennsylvania
Upinder Bhalla, California Institute of Technology
Avis Cohen, Cornell University
Nancy Kopell, Boston University
Rudolfo Llinas, New York University Medical Center
Michael Mascagni, NIH
Mark Nelson, California Institute of Technology
John Rinzel, NIH
Idan Segev, Hebrew University, Israel
Terrence Sejnowski, Salk Institute
John Uhley, California Institute of Technology
David Van Essen, California Institute of Technology
Christof Von Der Marlsburg, University of Southern California
Matthew Wilson, California Institute of Technology
Anthony Zador, Yale University
David Zipser, University of California, San Diego

Students
Ehud Ahissar, Hebrew University/Hadassah Medical School, Israel
Jeffrey E. Arle, University of Connecticut
Tony Bell, Vrije Universiteit Brussel, Belgium
Sherif Maher Botros, Massachusetts Institute of Technology
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Paul H. Frankel, Brown University
Merav Galun, Life Sciences Institute, Israel
Jisoon Ihm, Seoul National University, Korea
Dieter Jaeger, University of Michigan
Gilles Laurent, University of Cambridge, UK
Petra Leuchtenberg, University of Bonn, FRG
Patrick Lynn, University of Colorado, Boulder
Reinoud Maex, Katholieke Universiteit-Leuven, Belgium
Paul A. Moore, Boston University Marine Program/MBL
Valeriy Nenov, University of California, Los Angeles
Eduardo Solession, Syracuse University
Ehud Zoharry, Life Sciences Institute, Israel

Microinjection Techniques in Cell Biology
(May 21 to 26)

Director
Robert B. Silver, Cornell University

Faculty

Shinya Inoué, Marine Biological Laboratory
Douglas Kline, Ponce School of Medicine
Joanne Kline, Ponce School of Medicine
Katherine Luby-Phelps, Carnegie-Mellon University
Paul L. McNeil, Harvard Medical School

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Patricia L. Ansel, Dana-Farber Cancer Institute
Stuart K. Calderwood, Dana-Farber Cancer Institute
James A. DeCaprio, Dana-Farber Cancer Institute
Steven Demeter, University of Rochester
James V. Desirderio, Bristol-Myers Company, Connecticut
Frank J. Dye, Western Connecticut State University
Judith A. Eash, USDA, ARS, WRRC, PDP
Alison K. Hall, Case Western Reserve University
Seigo Izumo, Children's Hospital, Boston
Thomas B. Kinraide, USDA Agricultural Research Service
Thomas F. Lee, Saint Anselm College
Qun Lu, Emory University School of Medicine
James L. Madara, Harvard Medical School
Tadashi Maruyama, Marine Biotechnology Institute, Japan
John Ortega, University of Illinois
Ming Wan Su, SUNY Health Science Center, Brooklyn

Molecular Evolution (August 2 to September 1)

Directors

Mitchell L. Sogin, National Jewish Center for Immunology & Cell Biology
Mark Wheelis, University of California, Davis

Lecturers

Robert Cedegren, University of Montreal, Canada
Michael Clegg, University of California, Riverside
Bernard Davis, Harvard Medical School
Dan Davison, Los Alamos National Laboratories
Ford Doolittle, Dalhousie University, Canada
Jan Drake, NIEHS
Joseph Felsenstein, University of Washington
Walter Fitch, University of Southern California
Linda Goff, University of California, Santa Cruz
Richard Goldstein, Boston University School of Medicine
Michael Gray, Dalhousie University, Canada
James Lake, University of California, Los Angeles
Nancy Maizels, Yale Medical School
Lynn Margulis, University of Massachusetts, Amherst
Michael Melkonian, University of Cologne, FRG
Roger Milkman, University of Iowa
David Nanney, University of Illinois
Gary Olsen, University of Illinois
Colin Patterson, British Museum (Natural History), UK
Peg Riley, Harvard University
Eugene Small, University of Maryland
Temple Smith, Dana-Farber Cancer Institute
David Swofford, Illinois Natural History Survey
Bruce Walsh, University of Arizona
Alan Weiner, Yale Medical School
Jeffrey Wong, University of Toronto, Canada

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Debashish Bhattacharya, National Jewish Center for Immunology & Respiratory
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Robert G. Chapman, University of Massachusetts, Amherst
Zhi-Qing Chen, University of Alberta, Canada
Ivan E. Collier, Washington University School of Medicine
David S. Conant, Lyndon State College
Langtuo Deng, Memorial University of Newfoundland, Canada
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Keith O. Elliston, Merck Sharp & Dohme Research Laboratories
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Chris Gaskins, University of Oklahoma
Diddahally R. Govindaraju, Case Western Reserve University
Roger T. Hanlon, University of Texas
Linda K. Hardison, University of Washington
Richard E. Hudson, University of Arizona
Paul Joyce, Dalhousie University, Canada
Susanne R. Kaplan, Harvard University
Elizabeth Anne Kellogg, Harvard University Herbaria
Robert G. Kemp, Chicago Medical School
Anthony R. Kerlavage, NIH/NINDS
C. William Kilpatrick, University of Vermont
Ron S. Lundstrom, University of Utah

Ross J. MacIntyre, Cornell University
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W. Richard McCombie, National Institutes of Health
William S. Moore, National Science Foundation
Eva Y. Ng, Tufts University
Ftouhi Nouzha, University of Montreal, Canada
Brian Palenik, Massachusetts Institute of Technology
Bruno Paquin, University of Montreal, Canada
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Connie J. Wolfe, University of California, San Diego
Hong Xue, University of Toronto, Canada
Yinqing Yang, University of Houston
Karen Zeller, Smithsonian Institution

Optical Microscopy & Imaging in the Biomedical Sciences (March 5 to 11)

Directors

Nina Stromgren Allen, Wake Forest University
Colin S. Izzard, SUNY, Albany

Faculty

Kenneth A. Jacobson, University of North Carolina
John M. Murray, University of Pennsylvania
Kenneth Orndorff, Dartmouth College
Kenneth R. Spring, NIH/NHLBI
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Marty Bartholdi, Los Alamos National Laboratory
Gary Bird, NIH/NIEHS
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Jorgen Frokjaer-Jensen, The Panum Institute, University of Copenhagen, Denmark
Juyang Huang, Cornell University
Philip L. Huang, MGH/Harvard Medical School
Michael G. Klein, University of Maryland School of Medicine
Greg Law, Yale University School of Medicine
Raphael C. Lee, Massachusetts Institute of Technology
Denis A. Leong, University of Virginia Medical Center
Walter F. Mangel, Brookhaven National Laboratory
Babetta L. Marrone, Los Alamos National Laboratory
Elaine M. Merisko, Sterling Research Group, Dept. of Drug Delivery
Stewart Mullin, Carolina Biological Supply Company
Mailken Nedergaard, Cornell University Medical Center
Rudolf Oldenbourg, Marine Biological Laboratory
Hirotoshi Terada, Hamamatsu Photonics K.K., Japan

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Wheeler, George E., Brooklyn College
Wilbur, Charles, Colorado State University
Wittenberg, Beatrice, Albert Einstein School of Medicine
Wittenberg, Jonathan, Albert Einstein School of Medicine
Wolken, Jerome J., Carnegie Mellon University

Zimmerman, Morris, Watchung, NJ
Zipser, David, La Jolla, California
Zweig, Ronald, MBL

Domestic Institutions Represented

Alabama, University of, at Birmingham
Albert Einstein College of Medicine
American Bionetics, Inc.
Ames Laboratory
Analytical Luminescence Laboratory
Applied Biosystems
Arizona, University of
Arizona, University of, School of Medicine
Atlantex & Zieler Instrument Corporation
Axon Instruments, Inc.

Baruch College of CUNY
Baylor College of Medicine
Beckman Instruments, Inc.
Bethesda Research Labs
Bio-Rad Laboratories
Bodega Marine Station
Boston University
Boston University School of Medicine
Bowling Green State University
Brandeis University
Brigham & Women's Hospital
Brinkmann Instruments, Inc.
Brooklyn College of CUNY
Brown University

Bryn Mawr College
Bunton Instrument Company, Inc.

California Institute of Technology
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, Santa Cruz
Cambridge Instruments
Cambridge Technology
Carnegie-Mellon University
Case Western Reserve University
Chicago, University of
Ciba Corning Diagnostics Corp.
Cincinnati, University of
City College of New York
City University of New York (CUNY)
Clark University
Colgate University
Colorado College
Colorado, University of, Boulder
Columbia University
Columbia University College of Physicians and Surgeons
Connecticut, University of
Connecticut, University of, Health Center
Cornell University
Coy Laboratory Products
Crimson Camera Technical Sales

Dage MTI, Inc.
Dana-Farber Cancer Institute
Dartmouth College
Dawson Company
Delaware, University of
Diamond General Corporation
Digital Equipment Corporation
Donsanto Coporation
Duke University
Duke University Medical School

E. I. duPont de Nemours & Co., Inc.
East Tennessee State University
Eastman Kodak Company
E.G. & G., Inc.
Emory University
Emory University School of Medicine
Eppendorf, Inc.

Florida, University of
Flow Laboratories
Fotodyne, Inc.

General Scanning, Inc.
Georgetown University Medical School
Georgia, University of
Gilson Medical Electronics, Inc.
Gonzaga University
Grass Instrument Company

Hacker Instruments, Inc.
Frederick Haer & Company
Hahnemann Medical College
Harvard Medical School
Harvard University
Hawaii, University of
Health Sciences, University of
Holy Cross College
Honeywell Corporation
Howard Hughes Medical Institute
Howard University
Hunter College
Hunter College and CUNY New York Graduate Center

I.B.M.-T.J. Watson Research Center
ICN Radiochemicals
Illinois Natural History Survey
Illinois State University
Illinois, University of
Indec Systems, Inc.
Indiana University
International Biotechnologies, Inc.
Iowa, University of
Iowa, University of, College of Medicine
Institute for Basic Research in Developmental Disabilities
Institute of Neuroscience
ISCO, Inc.

JEOL
Johns Hopkins University School of Medicine

Kentucky, University of
Kentucky, University of, Medical School
Kinetic Systems
Kipp & Zonen
David Kopf Instruments

Lab Line Instruments, Inc.
Laser Science
Lehman College of CUNY
Lindberg Enterprises
Los Alamos National Laboratories
Loyola University
Ludlum Measurements, Inc.

Maryland, University of
Maryland, University of, School of Medicine
Massachusetts Institute of Technology
Massachusetts, University of, Amherst
Massachusetts, University of, Boston
Massachusetts, University of, Medical Center
Massachusetts, University of, Medical School
Medical College of Ohio
Medical Systems Corporation
Merck & Company, Inc.
Merck Sharp & Dohme Research Laboratory, New Jersey
Miami, University of
Miami, University of, School of Medicine
Michigan State University
Michigan, University of, Ann Arbor
Minnesota, University of
Molecular Probes
Mount Holyoke College
Murray State University

National Institutes of Health/NINDS
National Institutes of Health/NIEHS
National Institutes of Health/NIDDK
National Jewish Center for Immunology & Respiratory Medicine
National Science Foundation
Naval Medical Research Institute
Neslab Instruments, Inc.
New Brunswick Scientific Company, Inc.
New England Medical Center
New Jersey Medical School
New York Medical College
New York University Medical Center
Nikon, Inc.
North Carolina, University of, Chapel Hill
North Dakota University
Northwestern University Medical School

Oklahoma, University of
Olympus Corporation
Oregon State University
Oregon, University of

Pennsylvania State University
Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Perceptics Corporation
Perkin-Elmer Corporation

Pharmacia, Inc.
Pharmacia LKB Nuclear, Inc.
Photometrics, Ltd.
Photonic Microscopy, Inc.
Photon Technology International
Pittsburgh, University of
Pittsburgh, University of, Medical School
Polaroid Corporation
Pomona College
Ponce School of Medicine
Population Council-CBR, The
Portland State University
Princeton University

Quantex Corporation

Radiomatic Instruments & Chemical Company, Inc.
Reed College
Roche Institute of Molecular Biology
Rochester, University of
Rochester, University of, School of Medicine & Dentistry
Rockefeller University, The

Salk Institute
San Francisco State University
Sarastro, Inc.
Savant Instruments, Inc.
Scripps Clinic and Research Foundation
Sequoia Turner
Sony Medical Electronics
Southern California, University of
Stanford University
Stanford University Medical Center
State University of New York
State University of New York, Albany
State University of New York, Buffalo
State University of New York, Purchase
State University of New York, Stony Brook
State University of New York, Syracuse
Stratagene
Sutter Instrument Company
Swarthmore College
Swift Instruments, Inc.
Syracuse University

Technical Manufacturing Corporation
Technical Products International, Inc.
Technical Video, Ltd.
Temple University
Texas Christian University
Texas Tech. University
Texas, University of, Austin
Texas, University of, Medical Branch
Texas, University of, Medical School
Tufts University School of Medicine
Tufts University School of Veterinary Medicine
Tulane University
Turner Designs

Union College
United States Food & Drug Administration
Universal Imaging Corporation

Vanderbilt University
Vassar College
Videoscope International, Ltd.
Virginia, University of
Vital Images

Wake Forest University
Wake Forest University/Bowman Gray Medical School
Washington, University of
Washington University
Washington University School of Medicine
Wesleyan University
Wheaton College
Wild Leitz USA, Inc.
Williams College
Wisconsin, University of
Woods Hole Oceanographic Institution
Worcester Foundation for Experimental Biology

Yale University
Yale University School of Medicine

Zeiss, Carl, Inc.

Foreign Institutions Represented

Academy of Sciences, USSR

Basel, University of, Switzerland
Bergen, University of, Norway
Bielefeld, University of, FRG
Biozentrum, Basel, Switzerland
Bonn, University of, FRG
British Museum of Natural History, UK

Calgary, University of, Canada
Cambridge, University of, UK
Catania, University of, Italy
Chiba University, Japan
Chiba University School of Medicine, Japan
Chile, University of, C.E.C.S., Chile
CINEVESTAV-IPN, Mexico
Cologne, University of, FRG

Dalhousie University, Canada
Dalhousie University Medical School, Canada

Ecole Normale Superieure, France

Federal University of Pernambuco, Brazil
Freie Universitat, Berlin, FRG
Friedrich Miesener Institut, Switzerland

Goettingen, University of, FRG

Hebrew University, Israel

Imperial College of Science & Technology, UK

Institute for Biological Research, Yugoslavia
Instituto M. y. M. Ferreyra, Argentina
I.V.I.C., Venezuela

Katholieke Universiteit-Leuven, Belgium
Kobe University, Japan
Koln, University of, FRG
Konstanz, University of, FRG

Life Sciences Institute, Israel
London School of Hygiene & Tropical Medicine, UK
London, University of, Egham, UK

Max-Planck-Institut-fur-Biophysikalische Chemie, Nikolausberg, FRG

Max Planck-Institut, Gottingen, FRG
McGill University, Canada
Medical Research Council, UK
Milan, University of, Italy
Montreal, University of, Canada

Osnabruch, University of, FRG
Ottawa, University of, Canada

Philipps-Universitat, Marburg, FRG

Queens College, UK
Queens University, Canada
Queensland, University of, Australia

St. Andrews University, Scotland, UK
Seoul National University, Korea
Sienna, University of, Italy
Simon Bolivar, University of, Venezuela
State University of Utrecht, The Netherlands
Stazione Zoologica, Italy
Stockholm, University of, Sweden
Swiss Federal Institute, Switzerland
Swiss Federal Institute of Technology, Switzerland
Toronto, University of, Canada
Tubingen, University of, FRG

University College, UK
Universita di Palermo, Italy

Vrije Universiteit Brussels, Belgium

Wolfson College, UK

Z. L. F. Kantonsspital, Basel, Switzerland

Year-Round Research Programs

Boston University Marine Program

Faculty

Strickler, J. Rudi, Professor of Biology, Program Director
Atema, Jelle, Professor of Biology
Humes, Arthur G., Professor of Biology Emeritus
Tamm, Sidney L., Professor of Biology
Valiela, Ivan, Professor of Biology

Visiting faculty and investigators

Baldwin, Christopher, Boston University
Bloomer, Sherman, Boston University
Caballero, Pascual, University of Las Palmas, Gran Canaria
Chang, Patrique, Villefranche-sur-Mer, France
D'Avanzo, Charlene, Hampshire College
Finger, Thomas, University of Colorado
Gerhardt, Greg, University of Colorado
Kotrschal, Kurt, University of Salzburg, Austria
Marrase, Celia, University of Barcelona, Spain
Nakamura, Shogo, Toyama University, Japan
Peckol, Paulette, Smith College
Perez Castillo, Fernando, CIQRO, Cancun, Mexico
Peters, Rob, University of Utrecht, Netherlands
Peterson, Susan, Boston University
Rhoads, Donald
Richman, Sumner, Lawrence University
Rietsma, Carol, SUNY, New Paltz
Spungin, Ben, Tel-Aviv University, Israel
Stephens, Raymond E., Boston University School of Medicine
Tyack, Peter, Woods Hole Oceanographic Institution

Research staff

Costello, Jack, Research Associate
Foreman, Kenneth, Research Associate
Gerardo, Hortense, Research Associate
Tamm, Signhild, Senior Research Associate
Voigt, Rainer, Research Associate

Teaching assistants and staff

Hahn, Dorothy, Senior Administrative Secretary
Moore, Michael, Course Assistant
Moore, Paul, Course Coordinator
Mulsow, Sandor, Course Assistant
Saigh, Leila, Course Assistant
Sunley, Madeline, Administrative Manager

Graduate students

Alber, Merryl
Banta, Gary
Brewer, Matthew
Corotto, Frank
Coughlin, David
Cowan, Diane
Elskus, Adria
Gallager, Scott
Hersh, Douglas
Hwang, Jiang-shiou
Katz, Andrea
Kennedy, Blain
Gomez, George
LaMontagne, Michael
Lavalli, Kari
Mazel, Charles
Merrill, Carl
Moore, Paul
Mosiach, Simon
Mulsow, Sandor
Portnoy, John
Tamse, Armando
Trager, Geoffrey
Trott, Thomas
White, David
Varela, Diana

Undergraduate students Fall 1989

Abbot, Amy (Mt. Holyoke)
Anderson, Tami (Lawrence)
Argyros, George
Babione, Michelle (Hampshire)
Beneteau, Shana (Lawrence)
Bolles, Tom
Brown, Kevin
Calvo, Beatriz (University of Madrid)

Chau, Diane
Demenna, Ernest
Fernandez, Cecilia
Fitzsimmons, Maura
Getz, Jenna
Hoagland, Todd (Bucknell)
Hoban, Rona
Hough, Eric (Lawrence)
Hulbert, Nara (Lawrence)
Hunsicker, Karin (Wesleyan)
Kinkade, Chris
Kolberg, Kristin
Litt, Debbie (Miami, Ohio)
Lyford, Alison
MacDonald, Robin
Martin, Merrill (University of Puget Sound)
Modi, Jay (Bucknell)
Moran, Gretchen
Nakano, Takayuki
Nyberg, Jay (Lawrence)
Odien, Robert
Oppleman, Caroline
Pardo, Alex (Hampshire)
Pile, Adele
Poling, Kirsten
Pratt, John
Puria, Sushil
Sexton, Pamela
Snellman, Jane
Smith, J. Bailey
Sterling, Marie
Szulgit, Greg
Tremel, Don
Verhulst, Amy (Lawrence)
Weinstein, Anna

Summer undergraduate interns

Bergles, Dwight
Buckley, Joe
Casteline, Jennifer
Cochran, Wendy
Engelstein, Stefany (Yale)
Friedman, Eric
Lacomis, Lynne (SUNY, Binghamton)
McKnight, Andrew (University of Connecticut)
Melvin, Mary Kay
Michmerhuizen, Kate
Mintz, Joshua
Russell, Julie (Hampshire)
Rutka, Timothy
Sanders, Sophie (Dalton School)
Scholz, Nat
Short, Graham
Weinstein, Anna (Oberlin)

Winter undergraduate intern

Altes, Hester

Laboratory of Jelle Atema

Organisms use chemical signals as their main channel of information about the environment. These signals are transported in the marine environment by turbulent currents, viscous flow, and molecular diffusion. Receptor cells extract signals through various filtering processes. Currently, the lobster with its exquisite sense of taste and smell, is our major model to study the signal filtering capabilities of the whole animal and its narrowly tuned receptor cells. Research focuses on amino acids (food signals) and pheromones (courtship), neurophysiology of receptor cells, behavior guided or modulated by chemical signals, and computer modeling of odor plumes and neural filters.

Laboratory of Arthur G. Humes

Research interests include systematics, development, host specificity, and geographical distribution of copepods associated with marine invertebrates. Current research is on taxonomic studies of copepods from invertebrates in the tropical Indo-Pacific area, and poecilostomatoid and siphonostomatoid copepods from deep-sea hydrothermal vents and cold seeps.

Laboratory of Rudi J. Strickler

We use high-speed cinematography and special laser light optical systems with target tracking devices to observe zooplankton-algae, carnivorous-herbivorous zooplankton, and fish-zoo-plankton interactions. Lab and field results show the degree to which abiotic forces influence the evolution of species, feeding guilds, and predator-prey interactions. Additional topics in the feeding ecology of crinoids, bryozoans and other suspension feeding invertebrates enhance perception of the first consumer level in the aquatic food chain.

Laboratory of Ivan Valiela

Our major research activity involves the Waquoit Bay Land Margin Ecosystems Research Project. This work examines how human activity in coastal watersheds (including landscape use and urbanization) increases nutrient loading to groundwater and streams. Nutrients in groundwater are transported to the sea, and, after biogeochemical transformation, enter coastal waters. There, increased nutrients bring about a series of changes. The Waquoit Bay LMER is designed to help us to understand and model the coupling of land use and consequences to receiving waters, and to study the processes involved.

A second long-term research topic is the structure and function of salt marsh ecosystems, including the processes of predation, herbivory, decomposition, and nutrient cycles.

The Ecosystems Center

The Center was established in 1975 to promote research and education in ecosystems ecology. Eleven senior scientific staff and 40 research assistants and support staff study the terrestrial and aquatic ecology of a wide variety of ecosystems. These ecosystems range from northern Europe (trace gas emission from acid-rain affected forests) to the Alaskan Arctic (long-term studies of the controls of tundra, lake, and stream biota) to the Harvard Forest (long-term studies of the effects of disturbance in forest ecosystems) to Buzzards Bay (controls of anaerobic decomposition). Many projects, such as those dealing with sulfur transformations in lakes and nitrogen cycling in the forest floor, investigate the movements of nutrients and make use of the Center's mass spectrometry laboratory (directed by Brian Fry) to measure the stable isotopes of carbon, nitrogen, and sulfur. The research results are applied wherever possible to questions of the successful management of the natural resources of the earth. In addition, the ecological expertise of the staff is made available to public affairs groups and government agencies who deal with such problems as acid rain, ground water contamination, and possible carbon dioxide-caused climate change. Opportunities are available for postdoctoral fellows.

Staff and consultants

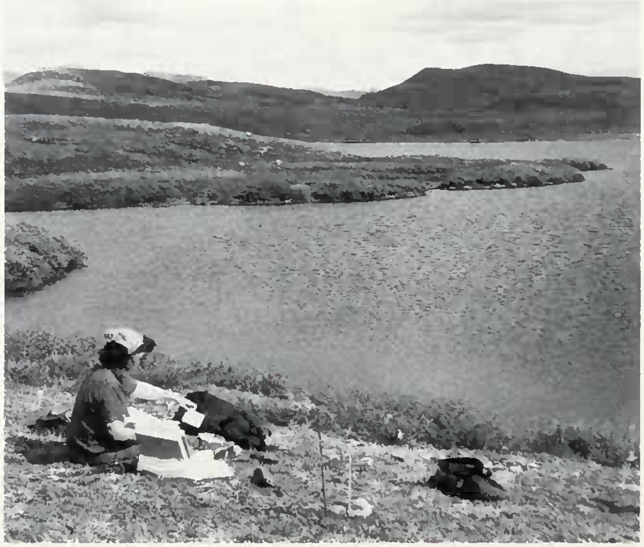
Hobbie, John E., Co-Director
Melillo, Jerry M., Co-Director
Banta, Gary
Bauman, Carolyn
Berger, Laurel
Boutwell, Anne
Bowles, Francis
Bowles, Margaret
Cochran, Wendy
Davis, Sarah
Danforth, Carolyn
Deegan, Linda
Dornblaser, Mark
Downs, Martha
Fafinski, Stephen
Fry, Brian
Garritt, Robert
Giblin, Anne
Griffin, Elisabeth
Helfrich, John
Hooper, David
Hopkinson, Charles
Hullar, Meredith
Jesse, Martha
Jordan, Marilyn
Kicklighter, David
Knudson, Heather
Kracko, Karen
Laundre, James
McKerrow, Alexa
Michener, Robert
Miliefsky, Michelle
Moller, Bernard
Nadelhoffer, Knute
O'Brien, Margaret
Pallant, Julie
Peterson, Bruce
Piterman, Oksana
Rastetter, Edward
Regan, Kathleen
Rhodes, Liesl
Ricca, Andrea
Russell, Ann
Saupe, Susan
Schwamb, Carol
Schwarzman, Beth
Semino, Suzanne
Shaver, Gaius
Steudler, Paul
Tucker, Jane

Postdoctorals

Bowden, Richard
Kling, George
Raich, James
Ryan, Michael
Wainright, Sam

Visiting scholars

Joyce, Linda, U.S.D.A. Forest Service
Neill, Christopher, University of Massachusetts, Amherst



Ecosystems staff member collects data from automated weather station at a lake at the headwaters of the Kuparuk River, Alaska.

Laboratory for Marine Animal Health

The laboratory provides diagnostic, consultative research, and educational services to the institutions and scientists of the Woods Hole community concerned with marine animal health. Diseases of wild, captive, and cultured animals are investigated.

Staff

Abt, Donald A., Director and The Robert R. Marshak Term Professor of Aquatic Animal Medicine and Pathology, School of Veterinary Medicine, University of Pennsylvania
Bullis, Robert A., Research Associate in Microbiology, University of Pennsylvania
Leibovitz, Louis, Director Emeritus
McCafferty, Michelle, Histology Technician, University of Pennsylvania
Moniz, Priscilla C., Secretary
Smolowitz, Roxanna, Research Associate in Pathology, University of Pennsylvania
Wadman, Elizabeth A., Microbiology Technician, University of Pennsylvania

Visiting investigator

Borkowski, Rosemarie, University of Florida

Laboratory of Aquatic Biomedicine

We study hematopoietic neoplasia, a leukemia-like disease of soft shell clams. Monoclonal antibodies developed by this laboratory and techniques in molecular biology are used to investigate the differences between normal and leukemic cells and their ontogeny.

Staff

Reinisch, Carol L., Investigator, MBL, and Chairperson, Department of Comparative Medicine, Tufts University School of Veterinary Medicine
Miosky, Donna, Laboratory Technician
Turano, Brian, Research Assistant

Laboratory of Cell Biochemistry

This laboratory studies developmental, metabolic, and environmental influences on the genetic regulation of cellular enzymes. Current emphasis is on the gene products involved in hepatic heme biosynthesis and utilization in marine fish. These processes are responsive to hormonal and nutritional signals as well as to environmental pollutants and carcinogens. This work is being conducted with fish liver *in vivo*, with primary cultures of normal hepatocytes, and with cultured hepatoma cells isolated from a fish tumor. Gene activity is quantitated with cDNA probes, and the relevant genes are being cloned in bacteria to define better the actions of chemical inducers. Other research is concerned with translocation of proteins between various subcellular compartments both in fish hepatocytes and in invertebrate eggs before and after fertilization.

Staff

Neal W. Cornell, Senior Scientist
Grace Bruning, Research Assistant
Helen Lee, Postdoctoral Fellow

Laboratory of D. Eugene Copeland

Electron microscopy of luminescent organs (photophores) in deep-sea fish; gas secretion in swimbladders of deep-sea fish; and osmoregulatory tissue in *Limulus*.

Laboratory of Developmental Genetics

This research group studies the early gene control of cellular differentiation pathways (cell lineage determination) in the embryos of tunicates and other marine invertebrate species.

Staff

Whittaker, J. Richard, Senior Scientist
Crowther, Robert, Research Assistant
Loescher, Jane L., Research Assistant
Meedel, Thomas H., Assistant Scientist

Visiting investigators

Collier, J. R., Brooklyn College
Lee, James J., Columbia University, College of Physicians & Surgeons

Laboratory of Judith P. Grassle

Studies on the population genetics and ecology of marine invertebrates living in disturbed environments, especially of sibling species in the genus *Capitella* (Polychaeta).

Staff

Grassle, Judith P., Senior Scientist
Mills, Susan W., Research Assistant

Laboratory of Harlyn O. Halvorson

This laboratory is interested in the molecular process of sporulation and spore germination in *Bacillus*, and in the regulation of phosphate metabolism in microorganisms.

We had earlier indentified several 1 genomic clones carrying the *gerJ* region of *Bacillus subtilis*. Subclones corresponding to *gerJ* were sequenced. The deduced amino acid sequence of the polypeptide does not show any homology with other *Bacillus* germination and sporulation genes that have been characterized, or with other bacterial genes. Spore germination requirements of several marine *Bacillus* species were also investigated. We found that these bacilli fell into two groups. The first responded only to germination stimulants characteristic of marine bacilli, while those in the second group responded to marine as well as terrestrial germination stimulants.

Inorganic phosphate transport in *Acinetobacter lwoffi* was further characterized. A high affinity transport system was identified which was repressed by high phosphate concentrations. Components of this system were lost by osmotic shock treatment, and activity could be recovered by incubation of shocked cells with concentrated shock fluid. The fact that shocked repressed cells did not respond to such treatment indicated that additional protein(s) are involved in high affinity phosphate transport.

Solubilization of rock (insoluble) phosphate by microorganisms has traditionally been attributed to be due to the metabolic formation of acids that help solubilize phosphate. Using several phosphate-solubilizing microbes, it was shown that "insoluble" phosphate was, in fact, sufficiently soluble to permit growth of organisms possessing high affinity phosphate transport systems

Staff

Halvorson, Harlyn O., Principal Investigator and Director, MBL
Chikarmane, Hemant, Assistant Scientist
Ferkowicz, Michael, Research Assistant
Pratt, Sara, Research Assistant

Visiting investigators

Anderson, Porter, University of Rochester
Keynan, Alex, Hebrew University, Jerusalem
Kornberg, Hans, Christ College, Cambridge
Vincent, Walter, University of Delaware
Yashphe, Jacob, Hebrew University, Jerusalem

Laboratory of Shinya Inoué

Mechanism of mitosis and related motility. Development of high resolution 3-D video microscope systems. High resolution polarized light microscopy of muscle fibrils. Physical origin of edge birefringence and image formation in the polarized light microscope.

Staff

Inoué, Shinya, Distinguished Scientist
Anniballi, Dyon, Programming Engineer
Berliner, Elise, Research Assistant
Boyd, Steven, Programming Engineer
Knudson, Robert, Instrument Development Engineer
Oldenbourg, Rudolf, Visiting Assistant Scientist
Taracka, Richard, Instrument Maker
Woodward, Bertha M., Laboratory Manager

Visiting investigators

Inoué, Theodore, Universal Imaging Corporation, Media, Pennsylvania
Silver, Robert B., Cornell University, Ithaca, New York

Laboratory of Molecular Evolution

The major research effort of this laboratory is the structure analysis of ribosomal RNA. Similarities between small subunit ribosomal RNA sequences are used to infer the evolutionary history of eukaryotic microorganisms and to design molecular probes for studies in marine ecology.

Staff

Sogin, Mitchell L., Director
Ariztia, Edgardo, Research Associate
Bhattacharya, Debashish, Post-Doctoral Fellow
Bibeau, Claude, Research Technician
Bucklin, Ann, Visiting Scientist
Elwood, Hille, Consultant
Stickel, Shawn, Research Technician

Laboratory of Neuroendocrinology

This laboratory studies the molecular and cellular bases of two neural programs that regulate different important behaviors in the model mollusc *Aplysia*. Research is conducted on the mechanisms of the neuronal circadian oscillators located in the eyes. These circadian oscillators drive the circadian activity rhythm of the animal, which is concerned with the daily timing of food gathering and of prolonged rest. Additional research is conducted on a group of neuroendocrine cells that produce a peptide, "egg-laying hormone," that initiates egg laying and associated behaviors. The laboratory is interested in how the three-dimensional shape of this peptide hormone allows a highly specific interaction with its receptor and the intracellular processes that are triggered by it. In another project, the laboratory has discovered and is continuing research on an anti-toxin protein that inhibits ADP-ribosylation of G-proteins induced by bacterial exotoxins.

Staff

Strumwasser, Felix, Director, Laboratory of Neuroendocrinology, MBL
Beetlestone, Linda, Laboratory Assistant
Cox, Rachel L., Senior Research Assistant
Glick, David, Senior Postdoctoral Fellow
Hellmich, Mark, Postdoctoral Fellow

Laboratory of Sensory Physiology

Since 1973, the laboratory has conducted research on various aspects of vision. Current studies focus on photoreceptor cells, on their light-absorbing pigments, and on their biochemical reactions initiated by light stimulation. Microspectrophotometric and biochemical techniques are used to study the receptors of both vertebrates (amphibia, fish, and mammals) and invertebrates (horseshoe crab and squid).

Staff

Harosi, Ference, Director, Associate Scientist, MBL, and Boston University School of Medicine
Szuts, Ete, Assistant Scientist, MBL, and Boston University School of Medicine
Trapp, Susan, Research Assistant

Visiting investigators

Cornwall, Carter, Boston University School of Medicine
Lall, Abner B., Johns Hopkins University
Miller, James L., University of California, San Francisco

Laboratory of Osamu Shimomura

Biochemical studies of the various types of bioluminescent systems. Preparation of the improved forms of aequorin for measuring intracellular free calcium.

Staff

Shimomura, Osamu, Senior Scientist, MBL, and Boston University School of Medicine
Shimomura, Akemi, Research Assistant

Visiting investigator

Nakamura, Hideshi, Harvard University

Laboratory of Raquel Sussman

Investigation of the molecular mechanism of DNA damage-inducible functions and structure-function relationships of γ repressor analyzed by immunological techniques. Present studies deal with the molecular mechanism of radiation-induced mutagenesis.

Staff

Sussman, Raquel, Associate Scientist
Cornuel, Catherine, Research Assistant
Wainwright, Patricia, Postdoctoral Research Associate

National Vibrating Probe Facility

We are exploring the roles of ionic currents, gradients, and waves in controlling development. We focus on controls of pattern and controls by calcium ions.

Staff

Jaffe, Lionel, Senior Scientist and Facility Director
Kuhtreiber, Wiel, Physiologist
McClaughlin, Jane, Laboratory Assistant
Miller, Andrew, Research Associate
Sanger, Richard, Technician
Shipley, Alan, Technician

Visiting investigators

Brownlee, Colin, Plymouth Marine Station
Buono, Mark, M.L.T.
Elliott, Ellen, Duke University
Fluck, Richard, Franklin & Marshall College
Isaacs, Hugh, Brookhaven National Lab
Kinraide, Tom, Appalachian Soil & Water Conservation Lab
Koshian, Leon, Cornell University
Lucas, William, University of California, Davis
McConnaughy, Ted, University of California, Davis
O'Donnell, Michael, McMaster University, Ontario, Canada
Rubinacci, Allessandro, University of Milano, Italy
Sardet, Christian, Station Marine Villefranche-sur-mer, France
Saunders, Mary Jane, University of South Florida, Tampa
Shankland, Martin, Harvard University
Smith, Peter, Cambridge University
Speksnijder, Annelies, University of Utrecht, The Netherlands
Zivkovic, Donna, University of Utrecht, The Netherlands

Honors

Friday Evening Lectures

Mary Lou Pardue, Massachusetts Institute of Technology, 30 June
"Heat Shock Locus 93D: A Gene with Unusual Functions"

Michael McElroy, Harvard University, 7 July
"Change in the Global Environment: Surprises from the Polar Stratosphere"

James Watson, Cold Spring Harbor Laboratory, 14 July
"The RNA Tie Club"

J. Murdoch Ritchie, Yale University School of Medicine, 20, 21 July (Forbes Lectures)
"Voltage Dependent Anion and Cation Channels in the Satellite Cells of the Nervous System" (20 July)
"A Pharmacologist's Approach to Multiple Sclerosis" (21 July)

Franklin M. Loew, Tufts University, 28 July
"The History, Science, and Politics of the Use of Animals in Research"

Robert B. Barlow, Jr., Syracuse University, 4 August (Lang Lecture)
"What the Brain Tells the Eye"

Leroy Hood, California Institute of Technology, 11 August (Monsanto Biotechnology Lecture)
"Biotechnology, the Genome Initiative, and Medicine of the Twenty-First Century"

Anthony Fauci, NIAID/NIH, 18 August
"Pathogenic Mechanisms of HIV Infection"

Terrence Sejnowski, Computational Neurobiology Laboratory, 25 August
"Computational Neuroscience: From Neurons to Networks"

Patricia Draper, Pennsylvania State University, 1 September
"Kung Bushmen: What the Study of Hunters and Gatherers Can Tell Us"

Fellowships

Robert Day Allen Fellowship

Fink, Rachel D., Mount Holyoke College

American Psychological Association Minority Fellowship Program in Neuroscience
July 17 to August 11

Directors
James Jones, American Psychological Association
Joe L. Martinez, Jr., University of California, Berkeley

Faculty
George M. Langford, University of North Carolina, Chapel Hill
Peter R. MacLeish, Rockefeller University
James G. Townsel, Meharry Medical College

Students
Dylan Bulseco
Bernard G. Crowell, Jr., Meharry Medical College
Ivy M. Dunn, University of Texas, Dallas
Katheryn L. Edwards, University of California, San Diego
Sonia I. Ortiz-Miranda, University of California, Davis
Weslia R. Patterson, University of Pennsylvania
Domingo Tomas Rivera, Clark University
Victor Sierra, St. John's University
Mary A. Tucker, University of Miami
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Constance Martyna
Nella McElroy
Lorraine Mizell
William Neall
Bertha Person
Julia Rankin
Lilyan Saunders
John Saunders
Elsie Scott
Deborah Senft
Charlotte Shemin
Marilyn Shepro
Cynthia Smith
Marguerite Smith
Judith Stetson
Barbara van Holde
Barbara Whitehead
Clare Wilber

MBL Tour Guides

Betsy Bang
John Buck
Sears Crowell
Teru Hayashi
Isabel Mountain
Julie Rankin
Lola Robinson
Donald Zinn
Margery Zinn

B Coolidge

Certificate of Organization
Articles of Amendment
Bylaws of the MBL

Certificate of Organization

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is
THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

Charles Endicott
Commissioner of Corporations)

Articles of Amendment

(On File in the Office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meeting of the corporation:

Voted: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

> "No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.
>
> "Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.
>
> "The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of $10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

Paul Guzzi
Secretary of the Commonwealth)

Bylaws of the Corporation of the Marine Biological Laboratory

(Revised August 11, 1989)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration or its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed with or without cause by the affirmative vote of two-thirds of the Trustees then in office. Any member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer, and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty-five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Members of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Trustees-at-large") approved by members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees-at-large shall be divided into four classes of *four*, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Trustees-at-large, shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti, who shall include any Member who has attained the age of seventy years (or the age of sixty-five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Trustees-at-large elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed ten. The number of Trustees-at-large so elected shall not exceed four and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six. Corporate Trustees shall always constitute a majority on the Board of those elected or approved by the Corporation.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting.

(F) A Corporate Trustee or a Trustee-at-large who has served an initial term of at least two years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as Trustee.

IX. (A) The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall annually elect a Treasurer who shall serve until his successor is selected and qualified. They shall elect a Clerk (a resident of Massachusetts) who shall serve for a term of four years. Eligibility for re-election shall be in accordance with the content of Article VIII(F) as applied to Corporate or Board Trustees. They shall elect Board Trustees as described in Article VIII(B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

(B) The Board of Trustees shall also have the power, by vote of a majority of the Trustees then in Office, to elect an Investment Committee and any other committee and, by like vote, to delegate thereto some or all of their powers except those which by law, the Articles of Organization or these Bylaws they are prohibited from delegating. The members of any such committee shall have tenure and duties as the Trustees shall determine; provided that the Investment Committee, which shall oversee the management of the Corporation's endowment funds and marketable securities, shall include the Chairman of the Board of Trustees, the Treasurer of the Corporation, and the Chairman of the Corporation's Budget Committee, as ex officio members, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business; but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director, and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. Beginning with the members elected for terms ending in 1990, one of the Trustees elected to serve on the Executive Committee should be a Trustee-at-large. This procedure will be repeated in the class of 1991, and henceforth the Trustees will elect to the Executive Committee Trustees to ensure that the composition of the Committee is four Corporate Trustees and two Trustees-at-large.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such subcommittees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may from time to time approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board of Trustees.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees, the Executive Committee, or any other committee elected by the Trustees shall constitute a quorum; and a lesser number than a quorum may adjourn any meeting from time to time without further notice. At any meeting of the Trustees, the Executive Committee, or any other committee elected by the Trustees, the vote of a majority of those present, or such different vote as may be specified by law, the Articles of Organization or these Bylaws, shall be sufficient to take any action.

XII. Any action required or permitted to be taken at any meeting of the Trustees, the Executive Committee or any other committee elected by the Trustees as referred to under Article IX may be taken without a meeting if all of the Trustees or members of such committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. The Trustees or members of the Executive Committee or any other committee appointed by the Trustees may also participate in meeting by means of conference telephone, or otherwise take action in such a manner as may from time to time be permitted by law.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such a manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. Except as otherwise provided below, the Corporation shall, to the extent legally permissible, indemnify each person who is, or shall have been, a Trustee, director or officer of the Corporation or who is serving, or shall have served, at the request of the Corporation as a Trustee, director or officer of another organization in which the Corporation directly or indirectly has any interest, as a shareholder, creditor or otherwise, against all liabilities and expenses (including judgments, fines, penalties and reasonable attorneys' fees and all amounts paid, other than to the Corporation or such other organization, in compromise or settlement) imposed upon or incurred by any such person in connection with, or arising out of, the defense or disposition of any action, suit or other proceeding, whether civil or criminal, in which he or she may be a defendant or with which he or she may be threatened or otherwise involved, directly or indirectly, by reason of his or her being or having been such a Trustee, director or officer.

The Corporation shall provide no indemnification with respect to any matter as to which any such Trustee, director or officer shall be finally adjudicated in such action, suit or proceeding not to have acted in good faith in the reasonable belief that his or her action was in the best interests of the Corporation. The Corporation shall provide no indemnification with respect to any matter settled or compromised, pursuant to a consent decree or otherwise, unless such settlement or compromise shall have been approved as in the best interests of the Corporation, after notice that indemnification is involved, by (i) a disinterested majority of the Board of Trustees or of the Executive Committee or, (ii) a majority of the Corporation's Members.

Indemnification may include payment by the Corporation of expenses in defending a civil or criminal action or proceeding in advance of the final disposition of such action or proceeding upon receipt of an undertaking by the person indemnified to repay such payment if it is ultimately determined that such person is not entitled to indemnification under the provisions of this Article XVI, or under any applicable law.

As used in this Article, the terms "Trustee," "director" and "officer" include their respective heirs, executors, administrators and legal representatives, and an "interested" Trustee, director or officer is one against whom in such capacity the proceeding in question or another proceeding on the same or similar grounds is then pending.

To assure indemnification under this Article of all persons who are determined by the Corporation or otherwise to be or to have been "fiduciaries" of any employee benefit plan of the Corporation which may exist from time to time, this

Article shall be interpreted as follows: (i) "another organization" shall be deemed to include such an employee benefit plan, including without limitation, any plan of the Corporation which is governed by the Act of Congress entitled "Employee Retirement Income Security Act of 1974," as amended from time to time ("ERISA"); (ii) "Trustee" shall be deemed to include any person requested by the Corporation to serve as such for an employee benefit plan where the performance by such person of his or her duties to the Corporation also imposes duties on, or otherwise involves services by, such person to the plan or participants or beneficiaries of the plan; (iii) "fines" shall be deemed to include any excise taxes assessed on a person with respect to an employee benefit plan pursuant to ERISA; and (iv) actions taken or omitted by a person with respect to an employee benefit plan in the performance of such person's duties for a purpose reasonably believed by such person to be in the interest of the participants and beneficiaries of the plan shall be deemed to be for a purpose which is in the best interests of the Corporation.

The right of indemnification provided in this Article shall not be exclusive of or affect any other rights to which any Trustee, director or officer may be entitled under any agreement, statute, vote of members or otherwise. The Corporation's obligation to provide indemnification under this Article shall be offset to the extent of any other source of indemnification or any otherwise applicable insurance coverage under a policy maintained by the Corporation or any other person. Nothing contained in this Article shall affect any rights to which employees and corporate personnel other than Trustees, directors or officers may be entitled by contract, by vote of the Board of Trustees or of the Executive Committee or otherwise.

XVII. There shall be no transfer of title or long-term lease of real property held by the MBL Corporation without prior approval of two-thirds of the full Board of Trustees. Such real property transactions shall be presented and discussed at one meeting of the Board and finally acted upon at a subsequent meeting of the Board. Either meeting could be a special meeting and no less than four weeks should elapse between these meetings.

Reference: *Biol. Bull.* **179**: 77–86. (August, 1990)

Hsr-omega, A Novel Gene Encoded by a *Drosophila* Heat Shock Puff*

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Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. Although originally identified because of its abundant transcription in heat shock, the *hsr-omega* gene is active, at generally lower levels, in non-stressed cells. The locus produces an unusual set of three transcripts. Evidence from a variety of experiments suggests that one of these transcripts acts in the nucleus, possibly to regulate the activity of a nuclear protein. Another of the transcripts appears to act in the cytoplasm, possibly monitoring or regulating some aspect of translation. The two transcripts together could have a role in coordinating nuclear and cytoplasmic activity. A number of processes occur in eukaryotic cells in which nuclear and cytoplasmic activities need to be coordinated; we suggest that *hsr-omega* plays a role in such coordination.

Introduction

Biologists have often found that a biological peculiarity in one organism can be exploited to study general questions that apply to many organisms. The polytene chromosomes that are found in some cells in *Drosophila* and some other organisms are examples of such a biological peculiarity. Studies of these chromosomes have contributed significantly to our understanding of how gene activity changes during development or in response to various agents (see Beermann, 1972; Hennig, 1987).

Polytene chromosomes are giant chromosomes, made up of many chromatids lying side by side in precise alignment. Although polytene chromosomes are condensed enough to allow cytological mapping, they are interphase chromosomes and thus allow us to actually see, *in situ,* chromatin structures involved in transcription and DNA replication. When a gene is being very actively transcribed, the site of that gene frequently undergoes a localized puffing of the many DNA strands that make up the chromosomes. Many transcribed regions do not make detectable puffs, but when a puff is seen, it is always a sign of very active transcription. A new puff indicates that transcription at the puff site has either been turned on, or turned up very sharply (Bonner and Pardue, 1977).

About 25 years ago, F. Ritossa (1962, 1964) found that when *Drosophila* larvae were placed at 37°C for a short period, nine new puffs were induced, suggesting that this heat shock induced nine new genes (Fig. 1). The genes were scattered over the chromosomes but still seemed to be controlled coordinately. Further, the same set of genes could be induced by other kinds of stresses, including a wide variety of chemicals. This induction was reversible; the puffs regressed as soon as the stress was removed. This was clearly a fascinating set of genes, but a good many years were to pass before it became technically possible to find out what the genes coded for. When that happened, the puffs were found to encode a small set of proteins now called the heat shock proteins, or hsps (see Ashburner and Bonner, 1979). The name "heat shock" is really a historical one because the proteins were first identified after a 37°C heat shock. We know now that these proteins are also induced by a variety of stresses and they are sometimes called the stress proteins. More recently it has become apparent that all of the hsps also have roles in non-stressed cells (for recent reviews on heat shock see Lindquist, 1986; Lindquist and Craig, 1988; Pardue *et al.,* 1988). In some cases, the hsps in non-stressed cells are encoded by the same genes that show increased activity in response to heat shock. In other cases, the hsps may be encoded by closely related genes, some activated by heat shock and some regulated in other ways.

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* This paper is based on a Friday Evening Lecture delivered at the Marine Biological Laboratory, Woods Hole, MA on 30 June 1989.

Present addresses: [1]Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6, [2]Eye Research Institute of the Retina Foundation, Boston, MA 02114, and [3]Department of Genetics, University of California, Berkeley, CA 94720.

After hsps had been identified in *Drosophila*, it became evident that all organisms make very similar sets of stress proteins. Animals, plants, and bacteria all show this heat shock response, so presumably the response has been around almost as long as cells have. The conservation of the major hsps is striking. For example, the hsp70 of *Drosophila* has 48% amino acid identity with the equivalent hsp of *E. coli* (Bardwell and Craig, 1984). Although the major proteins produced in the heat shock response are strongly conserved, the stimuli that induce the response vary from organism to organism and reflect the conditions under which the organism lives. For instance, the temperatures that induce heat shock in *Drosophila* cells are well below those that heat shock mammalian cells. This is not surprising because *Drosophila* cells usually live 10–20 degrees below the temperature of mammalian cells.

Evolutionary conservation argues that the heat shock response is very important, yet we know only a little about how it helps the organism. Clearly the response helps cells endure, for a short time, temperatures slightly above what they normally tolerate. If cells are subjected to a mild heat shock and make a low level of heat shock proteins and RNA, then they can survive temperatures that would kill them if they were moved directly to those temperatures.

We do not know how any of the hsps protect the cell from the heat shock, but studies of these proteins in nonstressed cells are showing that the hsps have very interesting roles in normal cells in addition to their roles in stressed cells. Three major families of hsps are now known to be conserved in plants, animals, and bacteria. For each of these families there is evidence suggesting that members act as "molecular chaperones." That is, these proteins appear to regulate the association of proteins with other macromolecules. The hsp70 family has many members; one of them has been implicated in translocation of secretory proteins into the endoplasmic reticulum and another appears to translocate proteins into mitochondria (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). The hsp 90 family (which includes the *Drosophila* hsp 82) appears to chaperone steroid hormone receptors and has also been found in association with some protein kinases (Catelli *et al.*, 1985). The bacterial hsp 70 is *dna K*, which is involved in protein-protein interactions in DNA replication (Georgopoulos *et al.*, 1989). A major bacterial hsp is *groEL*, which participates in bacteriophage assembly, although it probably has other roles (Herendeen *et al.*, 1979). Recently *groEL* has been shown to be related to an animal mitochondrial protein that is increased on heat shock (McMullin and Hallberg, 1988) and to a chloroplast protein that is involved in assembling the oligomeric enzyme Rubisco (Hemmingsen *et al.*, 1988).

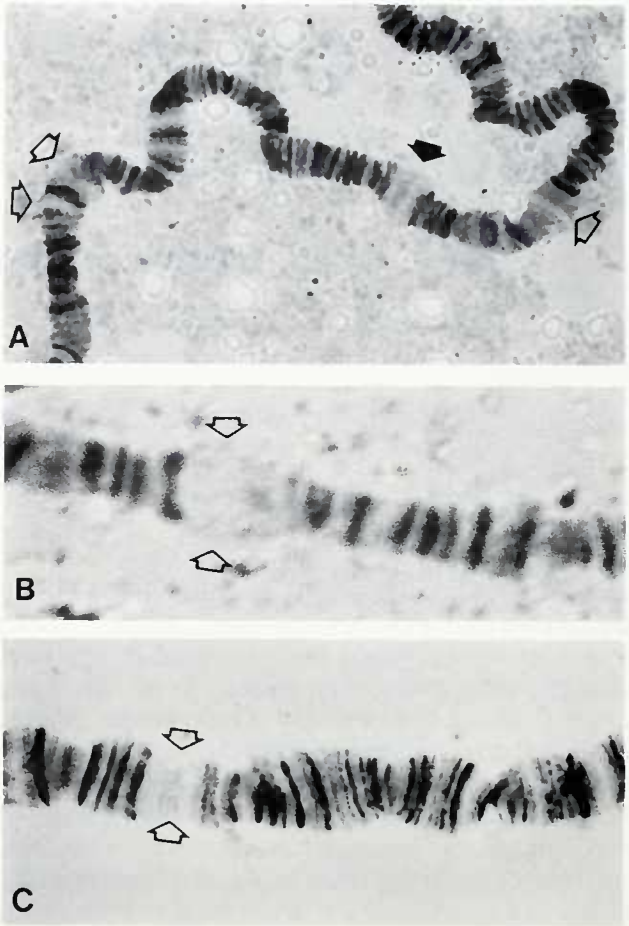


Figure 1. *Drosophila melanogaster* salivary gland chromosomes showing heat shock-induced puffing. (A) Part of chromosome 3 showing four of the major heat shock puffs (arrows, solid arrow indicates the 93D puff). × 900. (B) Higher magnification view of a 93D heat shock puff (arrows). ×1100. (C) The 93D region from a non-heat shocked larva. The region that puffs in heat shock is indicated by arrows. ×1100. The chromosomes in A and B are from larvae that had been heat shocked at 36°C for 30 min.

Thus the polytene puffs have revealed the existence of perhaps the most basic cellular response to stress and led to extensive study of this homeostatic mechanism. In turn, the studies of heat shock have accelerated our understanding of a new class of proteins, the chaperonins. The polytene puffs have still more to tell us. For instance, all of the known *Drosophila* heat shock protein genes have been found. As expected, the genes are in the heat shock puffs, but there are still a few heat shock puffs with no known function. One that has intrigued us especially is in region 93D in *D. melanogaster*.

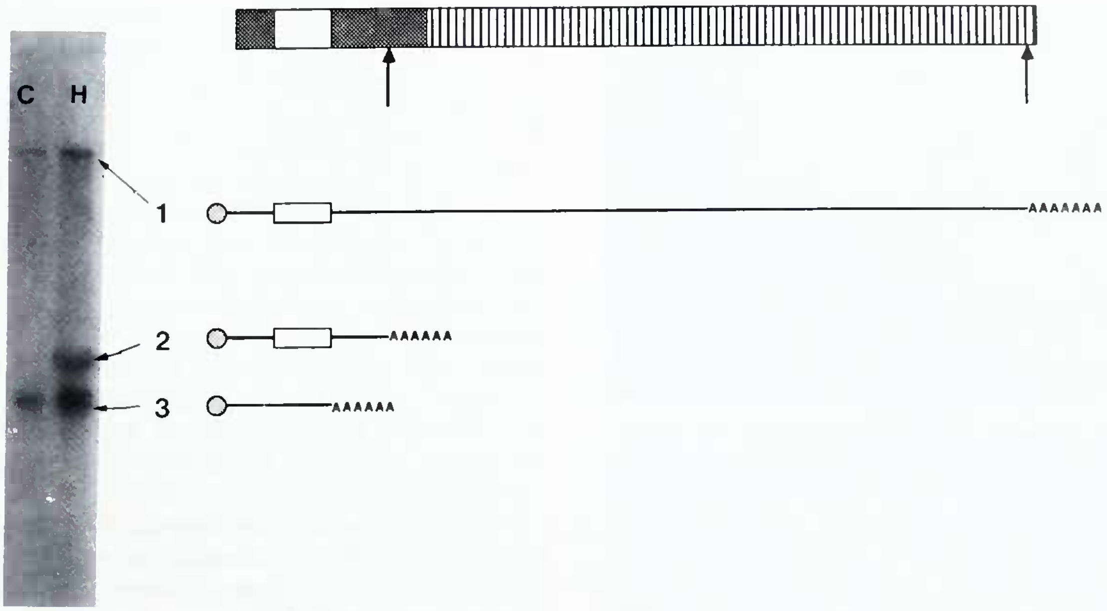


Figure 2. The transcripts of *hsr-omega*. The autoradiogram shows total RNA from control (lane C) and heat shocked (lane H) cultured *Drosophila* cells. The RNA has been gel fractionated, transferred to a filter, and hybridized with ^{32}P-labeled probe containing the sequence of transcript *omega 3* (and therefore complementary to a portion of the sequences of *omega 1* and *omega 2*, also). Longer exposures of the autoradiogram show that all three transcripts are present in the control cells (Garbe *et al.*, 1896). The top diagram shows the transcribed region of the gene. The shaded areas indicate the "unique portion" of the gene, much of which is incorporated in the cytoplasmic *omega 3* transcript. The white area represents the intron that is spliced out in the processing of *omega 3*. The striped area represents the region of small tandem direct repeats. The two arrows mark the polyadenylation signals used in the processing of *omega 2* (left arrow) and *omega 1* (right arrow). The three transcripts are diagrammed below the transcription unit.

The Unusual Puff at 93D

The puff at 93D is one of the very largest puffs, suggesting that it is actively transcribed during heat shock. Cytological studies have shown that 93D is unusual in a number of ways. (1) 93D is a bona fide member of the heat shock set, but it can also be induced by a number of agents that do not induce the other members of the family. (2) 93D contains large RNP granules never seen in other puffs. (3) 93D binds antibodies to several nuclear antigens not found in the other heat shock puffs. Every species of *Drosophila* studied has one (and only one) heat shock puff that has all these strange features (see Lakhotia, 1987).

The cytological studies strongly suggested that the unusual member of the heat shock puff set in each *Drosophila* species is homologous to the unusual member in each of the other species. We now have evidence that this is true and have named this puff the *hsr-omega* locus. (The name reflects its original identification as the locus encoding *h*eat *s*hock *R*NA *omega*, although we now think that the locus is important in almost all cells, whether or not they are heat-shocked.)

Although the homology story turned out happily in the end, it seemed for awhile that there was no sequence homology between these puffs. Berendes and his colleagues in The Netherlands isolated a cDNA clone from the *D. hydei* 2-48B puff (*i.e.*, the *D. hydei* heat shock puff with unusual features). The cDNA consisted of tandem 115 nt repeats (Peters *et al.*, 1984). The clone was used to select a cosmid clone of *D. hydei* DNA that covered the entire puff region. Neither the cosmid nor the cDNA showed any cross-hybridization with *D. melanogaster* DNA. Thus the 2-48B gene appeared to have no homologue in *D. melanogaster* DNA. However, we now know that 2-48B is the *D. hydei* homolog of *D. melanogaster* 93D (Garbe *et al.*, 1986). Apparently the sequence at this locus has evolved faster than the sequences of the other heat shock genes, whereas the phenotype, as deduced from cytological puffs, has been conserved. As discussed below, our studies of the 93D gene substantiate, and help to explain, this somewhat paradoxical conclusion.

We began our study of the 93D puff by using a cloned gene to determine the structure of the locus and its transcripts. The structure is quite different from that of the other heat shock genes or any other known gene (Fig. 2).

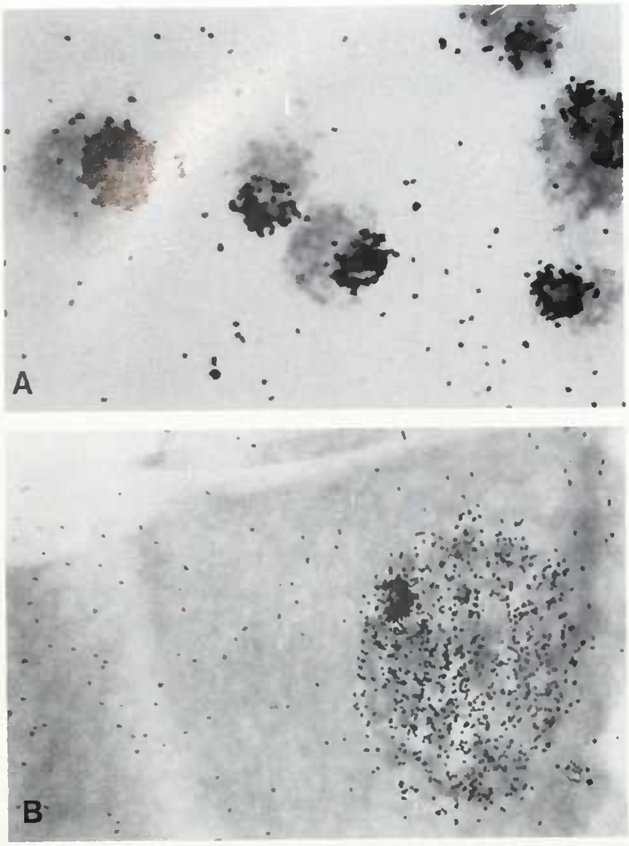


Figure 3. Autoradiograms showing the localization of *omega 1* to the nucleus in both diploid cells (A) and polytene cells (B). In both experiments the cells were fixed, permeablized, and hybridized with a ^{3}H-labeled probe complementary to the *hsr-omega* repeats (Bendena *et al.*, 1989a). The cells in (A) are cultured cells. ×1100. (B) shows the nucleus and part of the cytoplasm from a larval salivary gland cell. ×700. The cells in (A) have been heat shocked for 1 h at 36°C. The cell in (B) has not been heat shocked. Heat shock increases the amount of *omega 1* in the cell but does not affect the nuclear location.

The transcribed region is greater than 10 kb and can be divided into two parts: a 5′ unique region (approximately 3 kb) and a region of tandem repeats (8–25 kb, depending on the allele). These repeats are found nowhere else in the genome. Typical heat shock transcription signals can be found upstream from the start of transcription. Because the transcript is regulated in multiple ways, there must be other control signals also, but these have not yet been dissected (Garbe *et al.*, 1989).

In both normal and stressed cells, the *hsr-omega* locus produces a distinctive set of three major transcripts, all starting from the same nucleotide (Fig. 2). The largest transcript, *omega 1*, contains all of the sequence between the start and the second termination site (which is marked by a poly-adenylation signal). *Omega 1* is not a precursor for the other transcripts. It remains in the nucleus, and its turnover is controlled differently from the other two transcripts (Fig. 3). Its most remarkable feature is the segment of over 8 kb of tandem repeats. Each repeat is only 284 bp long, and the repeats differ from each other by less than 10% (Garbe *et al.*, 1986). The second 93D transcript, *omega 2*, appears to be made by an alternative termination near the first polyadenylation signal. *Omega 2* is also nuclear but it seems to be a more-or-less typical precursor that is spliced to make the cytoplasmic transcript, *omega 3*. These three transcripts have typical RNA processing sequences for splicing, polyadenylation, etc., but show no other similarities to known RNAs that might provide clues to function (Garbe *et al.*, 1989). We have, therefore, used a number of techniques—biochemical, cytological, and genetic (both classical and reversed)—to search for function (Bendena *et al.*, 1989). The results of these experiments will be discussed below.

Our studies to date suggest a working hypothesis about the function of the *hsr-omega* locus. Although not the only possible hypothesis, this seems to provide the simplest explaination of all the observations. Briefly, we suggest that *omega 1* serves to bind some component in the nucleus, thereby affecting either the activity or the level of this component in the nucleus. Because levels of *omega 1* vary quickly in response to cellular conditions, this binding would make the activity or the nuclear level of the bound component respond rapidly to those conditions and could thus serve a regulatory role. In the case of the cytoplasmic transcript, *omega 3*, we propose that the RNA, not a protein product, is important. One possibility is that the level of *omega 3* RNA reflects the rate of protein synthesis at any given time and provides a way to link another cell process to this rate. Finally, we wonder why both the nuclear and the cytoplasmic transcripts of *hsr-omega* come from the same start site. Does this in some way coordinate the initial levels of the nuclear and cytoplasmic transcript? A number of processes in eukaryotic cells have nuclear and cytoplasmic activities that must be coordinated. Perhaps this gene plays a role in such coordination.

Evolutionary Comparisons of *Hsr-omega* Genes

When we found that 93D had short tandem repeats, we were struck by its resemblance to the *D. hydei* 2-48B gene. Although the *D. melanogaster* repeats (284 bp) are larger than those in *D. hydei* (115 bp), such long stretches of repeats in a transcribed region were too unusual to dismiss as unrelated, notwithstanding the previous evidence that the genes shared no sequence homology. We used small pieces of the *D. melanogaster* DNA sequence to probe DNA from the cosmid clone of the *D. hydei* 2-48B locus. Our hybridization results confirmed those of Berendes and his colleagues, with one significant exception (Garbe and Pardue, 1986). Most fragments showed no cross-hybridization at any stringency. The small region that did show cross-hybridization, however, encouraged us to clone and sequence the rest of the *D. hydei* gene. The sequence analysis showed that the cross-hybridization was due to 60 nt of perfect homology and, surprisingly, 40 of the conserved nucleotides were in the intron, while the rest extended beyond the 3′ splice site. In spite of the differences in sequence, the *D. hydei* gene had the same structure as the *D. melanogaster* gene, and the location of the conserved region was the same in both genes. The same conserved sequence has now been found in the *hsr-omega* loci of all of the other *Drosophila* species that have been studied. The homology can be detected by *in situ* hybridization to polytene chromosomes. The sequence homology has been used to clone the *hsr-omega* gene from *D. pseudoobscura*. The three cloned genes enable us to compare *hsr-omega* sequences from distantly related *Drosophila*: *D. melanogaster* and *D. pseudoobscura* are separated by 46 million years, and both are separated from *D. hydei* by 60 million years (Beverley and Wilson, 1984).

Our studies of the *D. hydei* and *D. pseudoobscura* genes have shown that the structure of the *hsr-omega* locus is conserved, although the sequences have diverged (Garbe *et al.*, 1988). In each *Drosophila* species, the locus has a unique region followed by a long string of tandem repeats. In each species, there are three transcripts of approximately the sizes found in *D. melanogaster*. In each species the 60 nt conserved region is in the intron and overlaps the 3′ splice site.

In spite of the strong evolutionary divergence, the sequence comparisons give useful clues about possible functions. The clues are strong because the evidence that so much of the sequence can change increases the significance of the parts that have not changed.

The unique portion of the gene

In any pairwise alignment of the sequences from *D. melanogaster*, *D. hydei*, or *D. pseudoobscura*, the longest conserved sequence is the 60 nt around the 3′ splice site (Garbe *et al.*, 1988). (The conserved region rises to 62 nt when the *D. melanogaster* sequence is compared with that of *D. pseudoobscura*.) This conserved sequence might be necessary for splicing in heat shock; however that seems unlikely if one considers a similar sequence comparison of the *hsp83* gene. The hsp83 transcript is also spliced in heat shock and it shows very little sequence conservation in the intron (Blackman and Meselson, 1986). The hsp83 exon shows significant sequence conservation, but because the exon codes for protein, it is probably the protein sequence that is conserved, an explanation that does not hold for the 93D exon, which does not encode a protein. At this point, the reason for this conserved sequence is a puzzle.

With the exception of the 60–62 bp conserved region, pairwise alignments of the rest of the unique region of *hsr-omega* show few stretches of conserved sequences longer than 10–20 bp. Interestingly, some of the longest regions of homology surround sites that our RNA studies indicate to be important for RNA processing and function. These include 14–16 bp (depending on the species compared) at the 5′ splice site, and 15–21 bp at the polyadenylation signal. In addition, the transcription start site shows conservation between the species and also has five of the six specific nucleotides that are conserved in all *Drosophila* heat shock mRNAs, except the hsp83 mRNA. The sequence alignments also show why there is so little cross-hybridization between *hsr-omega* genes. There have been many short insertions and deletions that eliminate runs of sequence long enough to hold a hybrid. The deletions and insertions tend to balance out, so the sizes of the exons and introns are conserved.

Another conserved feature of the *hsr-omega* genes is the lack of long open reading frames (Garbe *et al.*, 1986). The only open reading frames (ORFs) are very short (shorter than those found on the opposite, non-transcribed strand). A comparison of the transcripts from the three *Drosophila* species shows only one ORF that is at all conserved in location or sequence (Garbe *et al.*, 1989). The location is interesting because, in each species, this ORF is the first one that is in a sequence context thought to be favorable for translation. The sequence conservation is not very strong; only the first four amino acids, plus a few other scattered amino acids, would be the same in all three translation products. Even the size of the ORF varies; the translation product would contain 23, 24, and 27 amino acids, depending on the species. In spite of the low level of conservation, other studies (discussed below) strongly suggest that this ORF is important in the function of the *omega 3* transcript.

The general conclusion from these sequence studies on the unique part of the gene seems to be that the cell is conserving the ability to make, splice, and polyadenylate an RNA of this size. This conclusion is based on the small conserved regions, the significance of which we already know. There are other small conserved segments that we cannot now decode; these regions probably also have functional importance.

The tandemly repeated segment of the gene

Restriction enzyme mapping of DNA from *hsr-omega* loci indicates that, in all *Drosophila* species, this locus has >8 kb of short tandem repeats. Repeats from both *D. melanogaster* and *D. hydei* have been sequenced. Within each *Drosophila* species the repeats show <10% divergence, but between species the repeats differ in both size and sequence. There is, however, a conserved 9 nt segment, AUAGGUAGG, that is found once in the 115 nt repeat of *D. hydei* and twice in the 284 nt repeat of *D. melanogaster* (Garbe *et al.*, 1986). The 9 nt segment thus occurs at about the same frequency along the transcripts. *D. pseudoobscura* repeats have not yet been sequenced, but preliminary evidence suggests that they will also have the 9 nt sequence. The sequence, AUAGGUAGG, is in the size range of sequences that have been shown to serve as binding sites for proteins and RNAs. The sequence may serve as a binding site in the *omega 1* RNA. If so, the repeats may be a device to maintain a certain number and spacing of copies of the binding site.

The evolution of the *hsr-omega* repeats appears much like that of satellite DNA. That is, the repeats are very homogeneous within each species, but diverge rapidly between species. The *D. melanogaster* repeat will hybridize only with DNA from the sibling species, *D. simulans*, and, even in this case hybrid stability is reduced, and restriction site differences indicate some sequence change.

The Cytoplasmic Transcript, *Omega 3*

The cytoplasmic RNA, *omega 3*, is spliced and polyadenylated, two characteristics usually associated with mRNAs, yet the only open reading frames (ORFs) are small and show very little conservation. Surprisingly, *omega 3* is found on polysomes (monosomes and disomes) by all known criteria (Fini *et al.*, 1989). The localization in control cells does not change when the cells are heat shocked. In both cases almost all of the transcript was loaded on the polysomes; rarely is any of the transcript free. In control cells, *omega 3* turns over rapidly but is stabilized by all inhibitors of protein synthesis (Fig. 4) (Bendena *et al.*, 1989). There is now evidence that turnover of certain mRNAs is linked to their presence on polysomes (Hunt, 1988). Although the studies described below suggest that *omega 3* is not an mRNA, *omega 3* shows a specific and rapid turnover when it is associated with active polysomes, as do these mRNAs (Bendena *et al.*, 1989b).

These evidences of an association between *omega 3* and protein synthesis have led us to search very hard for an *hsr-omega* translation product (Fini *et al.*, 1989). That search has been unsuccessful; but we have obtained indirect evidence that the small, partially conserved, ORF in *omega 3* is translated. The indirect evidence comes from experiments with recombinant DNA molecules in which a bacterial chloramphenicol acetyltransferase (CAT) gene was joined to the 5′ part of the *hsr-omega* sequence (Fig. 5). The constructs were stably transformed into *Drosophila* cultured cells and tested for their ability to direct synthesis of mRNA and CAT protein. The experiments showed that *omega 3* ORF blocks translation of a CAT gene placed just 3′ to it, as long as the *omega 3* ORF has a termination codon so that the ribosome must reinitiate in order to translate CAT. In contrast, when the *omega 3* ORF is fused in frame to CAT, a CAT protein of appropriately larger size is produced. These transformation experiments give evidence that the *omega 3* ORF is translated *in vivo*, although no product can be detected. Thus the product of the ORF must either be degraded or be sequestered very rapidly. We think the first alternative is most likely because we have been unable to find any evidence of the product, even in the transformed cells where the excess product might be expected to saturate or slow a sequestration mechanism (Fini *et al.*, 1989).

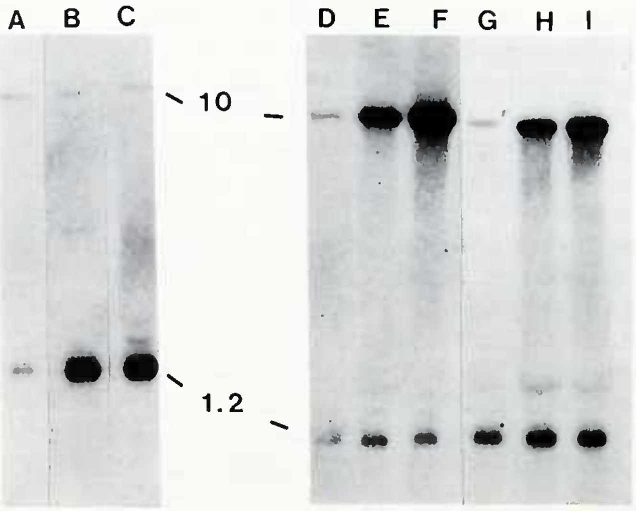


Figure 4. Autoradiograms showing differential regulation of the nuclear and cytoplasmic transcripts of *hsr-omega*. Inhibitors of protein synthesis lead to preferential accumulation of the cytoplasmic transcript, *omega 3*. Drugs that induce puffing of 93D without inducing other heat shock loci (*e.g.*, colchicine, benzamide) lead to preferential accumulation of the nuclear transcript, *omega 1*. To make the autoradiogram, RNA from cultured Drosophila cells has been fractionated and probed with the *omega 3* sequence, as in Figure 2. RNA from control cells (A, D, G), cells treated for 2 h with 10^{-4} *M* cycloheximide (B), cells treated for 2 h with 10^{-7} *M* pactamycin (C), cells treated with 10 m*M* benzamide for 12 h (E) or 24 h (F), cells treated with 100 μg/ml colchicine for 12 h (H) or 24 h (I). The 10 kb *omega 1* and the 1.2 kb *omega 3* transcripts are indicated.

Taken together, the studies on *omega 3* have suggested the following working hypothesis: the translation of the *omega 3* ORF functions to allow a polysome-associated turnover of *omega 3* and this turnover in some way serves to monitor, or regulate, some aspect of protein synthesis in the cell. Possibly, the *omega 3* RNA, or a degradation product of this RNA, has a function with a rate determined by the rate at which *omega 3* turns over on the polysomes. Admittedly this is highly speculative, but polysome-associated turnover may be common for RNAs that can be turned over rapidly (Hunt, 1988). The turnover of *omega 3* would reflect the level of protein

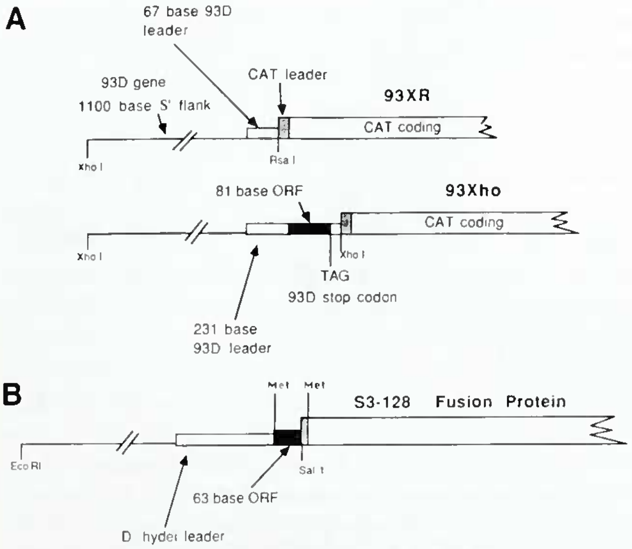


Figure 5. Recombinant DNA constructs used to analyze the translation of the most conserved small open reading frame (ORF) in *hsr-omega.* (A) The 93Xho construct was made by joining a bacterial chloramphenicol acetyltransferase (CAT) gene to DNA from the *D. melanogaster hsr-omega* gene. The junction was 30 nucleotides past the termination of the small ORF. The 93XR construct was made by deleting the small ORF from 93Xho. If the small ORF is translated *in vivo,* the ribosomes would not be expected to reinitiate on the CAT gene. Therefore the 93Xho construct would not be expected to direct the synthesis of CAT while the 93XR construct, where the CAT translation start is not blocked, should. When the constructs were stably transformed into *Drosophila* cultured cells, both constructs yielded abundant RNA but only the 93XR CAT gene was translated. (B) The S3-128 construct was made using the *D. hydei hsr-omega* gene. In this case the bacterial CAT gene was joined in frame to the conserved ORF so that ribosomes translating the ORF should continue translating into the CAT gene and yield CAT protein that is larger by the size of the ORF peptide. Cells carrying this construct do produce the predicted larger CAT. All of the experiments give strong evidence that the *hsr-omega* ORF is translated *in vivo* (Fini *et al.,* 1989).

synthesis at any particular time and could link some other cellular process to this level.

The Nuclear Transcript, *Omega 1*

One of the questions that we had about *hsr-omega* was whether all of the agents that induced the puff were acting on the *hsr-omega* sequences. Puffs usually involve more DNA than is actually transcribed, so a puff in the 93D region might indicate activation of a transcription unit that is not *hsr-omega.* The question had interesting implications. If the agents that induce the *hsr-omega* puff, but not the rest of the heat shock puffs, were inducing the transcripts that we are studying, it would suggest that *hsr-omega* is more sensitive to its environment than the other heat shock loci. Other experiments had shown that treatments inducing a puff at 93D blocked induction by a second agent, if the two inducers are applied in a relatively short time (Lakhotia, 1987). If all the inducers were acting on the *hsr-omega* gene, the observation that one inducer blocks activation by a second agent suggests that the *hsr-omega* locus can autoregulate, with products of the first induction inhibiting later induction. We have tested several inducers (Bendena *et al.,* 1989) and have found that all of the inducers do act on the *hsr-omega* locus (Fig. 4). These results suggest a responsiveness to external agents and an autoregulation that could characterize a regulatory locus. This sensitivity to the environment is consistent with the rapid response seen in heat shock in which the 93D region puffs slightly before the other loci and also returns to control levels more rapidly when cells are returned to normal temperature (Fig. 6).

These experiments with the other inducers gave an unexpected result. The level of *omega 1,* but not *omega 2* or *omega 3,* is increased by the agents that induce the 93D puff without inducing the rest of the heat shock loci (Fig. 4). The level rises rapidly in response to the agent, remains high so long as the agent is present, and drops rapidly when the agent is removed. Thus, the level of *omega 1* at any time reflects something that the cell perceives in its environment. The *omega 1* transcript accu-

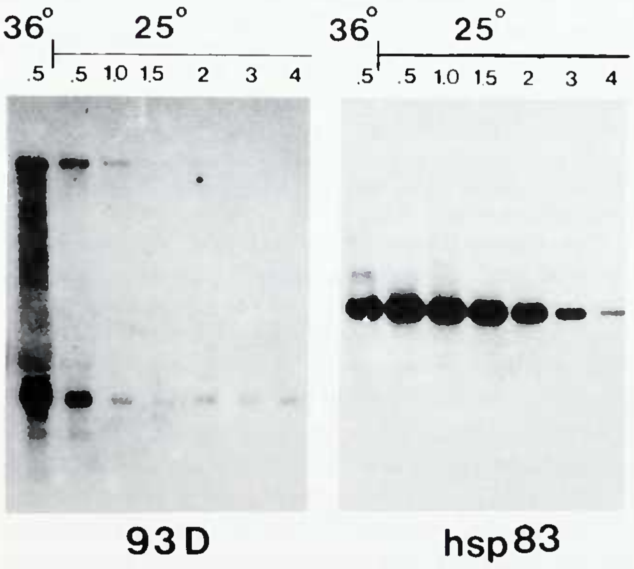
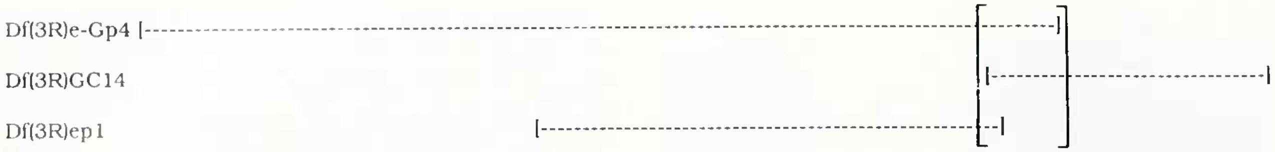


Figure 6. Autoradiogram showing that *hsr-omega* transcripts return to control levels more rapidly than the hsp83 transcripts upon recovery from heat shock. Cultured *Drosophila* cells were heat shocked for 30 min at 36°C and then returned to 25°C. Aliquots were taken at times indicated above each lane and the RNA was analyzed as in Figure 2. The panel marked 93D shows the *hsr-omega* transcripts as detected by the probe for the *omega 3* sequence. The panel marked hsp83 shows the same samples probed for the sequences encoding the D. melanogaster hsp83. The *hsr-omega* transcripts return to control levels at 1–1.5 h while the hsp83 transcripts do not return to control levels until 4 h after return to 25°C. (The autoradiogram shows only samples taken up to the time at which hsp83 returned to control levels.)



(er7	er4)	(er6)	(er5)	(er14)	(er2	e)	(er1)	(er8	er11	er9)	(er3)	(er19	er13	er16)
EC3	GC15	GC20	GC18	DC1	GC11		GC8	EC13	EC36	GC23		GC30	EC40	GC27
EC4	GC16	EC7	GC19		EC21		GC31	EC17	EC38	EC15			EC43	EC44
EC8	GC17	EC11	GC21		EC24		EC19	EC28						
EC9	EC2		EC1				EC20	EC32						
EC10	EC16		EC18				DC3	EC37						
EC30	EC25		EC26											
	EC27													
	EC33													

Figure 7. Diagram of the chromosomal deletions that bracket the *hsr-omega* locus and of the lethal complementation groups in that region in *D. melanogaster*. The lines indicate the relative locations and sizes of the three deletions. The brackets indicate the region of overlap of Df(3R)e^{Gp4} and Df(3R)GC14, which contains the *hsr-omega* locus. The complementation groups are listed below in order. In those cases where the order has not been established, the complementation groups are enclosed in parentheses. Below the name of each complementation group are given the names of the individual mutations that have been recovered in that group. The region of overlap of Df(3R)e^{Gp4} and Df(3R)GC14 is designated complementation group er3; it appears to contain only the *hsr-omega* locus but this has not been formally proven. Animals carrying both deficiencies and, therefore completely lacking er3, grow very poorly and do not survive to adulthood. Thus er3 appears to be an essential locus, but no point mutations in er3 have been recovered. Complementation group e is the *ebony* locus; several mutations in this group were recovered but were not scored. Because multiple mutants have been recovered in most of the complementation groups, it appears that er3 is unusually resistant to mutagenesis. Df(3R)ep1 removes the heat shock control elements, but not the constitutive activity of *hsr-omega*. Animals carrying this deletion and Df(3R)GC14 are fully viable at optimum temperatures but are much more sensitive to slightly increased temperatures than wild type flies.

mulates around the nucleus, apparently just under the nuclear membrane (Fig. 3).

The striking feature of the *omega 1* sequence is the array of tandem repeats containing an evolutionarily conserved potential binding site for a protein or RNA. The idea that the *hsr-omega* transcript binds protein is attractive because one of the distinctive features of the locus is the presence of 300 nm RNP granules not seen at any other locus. These granules could be formed by *omega 1* and its bound protein. This structural evidence, coupled with the evidence that levels of this RNA can change rapidly, suggests that *omega 1* might sequester variable amounts of a protein or RNA in response to variable cell conditions. The binding might affect the activity of the bound agent, either positively or negatively, or it might simply change the concentration that is present in the nucleus. In any case, such binding could allow *omega 1* to play a regulatory role for some aspect of cell metabolism. One way to test this hypothesis is to try to find a molecule that specifically binds to the *omega 1* RNA. We have recently found that an *in vitro* RNA transcript containing only the repeats from *omega 1* binds specifically to three bands on a blot of gel-fractionated *Drosophila* proteins: one band migrates at about 170 kD and a doublet migrates at 53–55 kD. The bands could represent a set of related proteins; alternatively, the smaller doublet could represent monomers or degradation products of the 170 kD band. These studies are preliminary, but the characterization of the proteins represented by these gel bands should give us useful information about the function of *omega 1*.

Genetic Analyses of *Hsr-omega*

Our genetic studies (Mohler and Pardue, 1984) are consistent with the hypothesis that *hsr-omega* has some sort of regulatory role. We have identified two chromosomal deletions, e^{Gp4} and GC14, that completely remove the *hsr-omega* sequences. e^{Gp4} removes 11 complementation groups proximal to *hsr-omega*, and GC14 removes 3 complementation groups distal to *hsr-omega*, but the 2 deficiencies appear to overlap only for *hsr-omega* (Fig. 7). Extensive attempts to recover point mutations in the region of overlap gave no lethal, visible, or temperature-sensitive mutations. What we know about the sequence conservation of *hsr-omega* suggests that it would be almost impossible to inactivate it by a point mutation (or even by a tiny deletion), so we are not surprised to get no *hsr-omega* mutants. The genetic evidence provides a strong argument that no other genes (of conventional mutability) are located in the region of overlap. If e^{Gp4}/GC14 heterozygotes are indeed homozygous deficient only for *hsr-omega*, then their phenotype indicates that *hsr-omega* is important in non-stress situations. These e^{Gp4}/GC14 heterozygotes hatch as well as normal sibs (but mothers had one *hsr-omega* gene) but grow very slowly, dying at all stages of development. None survive as adults.

More recently we have recovered another deletion, ep1, that removes six complementation groups proximal to *hsr-omega* and also deletes the heat shock transcription signals, but not the constitutive transcription signals or the transcribed sequence of *hsr-omega* (Garbe, 1988). Heterozygous ep1/GC14 flies are viable and fertile at normal growth temperatures (25°C) but cannot grow at 31°C, a temperature at which wild type flies grow (but do not make sperm). The simplest explanation of the analysis of these two heterozygotes is that e^{Gp4}/GC14 flies die because they lack any *hsr-omega* gene, and the ep1/GC14 flies die at 31°C because their only *hsr-omega* gene has no heat shock control. But it is formally possible (considering the two results together) that the overlap of e^{Gp4} and GC14 also includes a lethal gene distal to *hsr-omega*, while the overlap of ep1 and GC14 includes a temperature-sensitive gene proximal to *hsr-omega*. (The other possibility, that either phenotype is simply due to the sum of the hemizygous loci, was excluded by testing animals carrying larger deficiencies for this region.) We have mapped the breakpoint of the ep1 deficiency and find that it is just downstream of the closest heat shock transcription signal of *hsr-omega*. The breakpoints of the other two deficiencies lie outside the region that we have cloned (about 5 kb 5′ and about 1 kb 3′). The ep1 deletion shows that there is no undiscovered lethal gene 5′ to *hsr-omega* that could cause death of the eGp4/GC14 heterozygotes. (If there were, ep1/GC14 flies would also die.) A highly repeated element just past the 3′ end of *hsr-omega* has made walking in that direction difficult, and we have not pursued it because breakpoint mapping cannot definitively reveal whether the phenotype we observe is due to the *hsr-omega* gene or to some other gene. The proof must be genetic. Our mutagenesis experiments were extensive, and they detected no mutable gene in this region. Thus, the genetic evidence argues that *hsr-omega* is the only gene in the overlap region; however, the definitive test must be rescue of the mutants by P-element transformation with the cloned gene. In the meantime, we suggest that, if the two transheterozygote phenotypes are due to *hsr-omega*, then they are consistent with a regulatory role for this locus. One possible type of regulatory role would be to link two processes in a way that increases metabolic efficiency. Mutants lacking the regulator would have the two processes running freely, at considerable expense of energy. Such mutants might grow slowly and have trouble making it past crucial developmental points or surviving in sub-optimal environments, as do the putative *hsr-omega* deletions. Because *hsr-omega* produces both nuclear and cytoplasmic transcripts, we speculate that the locus acts to link a nuclear with a cytoplasmic process.

Conclusion

We now have several kinds of information about the *hsr-omega* locus. It is clearly different from other genes, and some of those differences might explain why the locus has not attracted attention before. It seems to be relatively insensitive to mutagenesis and so would not be easily picked up in genetic studies. (Non-protein coding genes may be generally less sensitive to mutagenesis because, while any frame-shift or stop codon can destroy the function of an mRNA, RNAs with other functions are not so strongly polar as mRNAs; many small deletions or base changes in a non-coding RNA may make small local perturbations that do not affect function.)

Like the heat shock response, which was identified from polytene puffs, the *hsr-omega* locus may be found in many organisms. Because the sequence is evolving so rapidly, it may not be possible to use hybridization to detect the DNA in other genera, and the search for the gene in other organisms must wait until the function is better understood. Nevertheless, we think that this locus, like the loci encoding hsps, is common to many organisms.

Acknowledgments

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Unisex Flash Controls in Dialog Fireflies

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Abstract. During courtship in many dialog fireflies, the female flashes at a fixed interval after each rhythmic display signal of the male. The male then orients toward her flash, but does not flash in response. In three species, males also may decoy other males by answering them after an interval equal to the female's characteristic flash delay. In two other species, individuals have been induced to respond to, or to duplicate, interflash intervals characteristic of the opposite sex. Both male and female thus harbor overt or latent homologs of some of the other's flash-timing circuits.

Introduction

The lock-and-key nature of many courtship communication systems shows that one sex has a suite of behavioral controls that fit those of the other. Quantitative interdigitation of stimulus and response is perhaps nowhere shown more starkly than in the timed dialogs of certain fireflies. In *Photinus pyralis,* for example, the flying male emits spontaneous advertising flashes at about 6-s intervals, hovering for about 2 s after each flash. The sedentary female flashes only responsively, about 2 s after seeing a flash of the male. If the male sees a flash 2 s after his flash, he flies toward it. The characteristic 2-s response delay of the female is the necessary and sufficient signal for the orientation of the male (Buck, 1937). Therefore, the nervous system of the male undoubtedly contains an endogenously activated, 2-s window-opening circuit that is tuned to the visually activated 2-s flash-control circuit of the female.

In typical time-coded courtship dialogs, the female is the responder and the male the advertiser. The male ordinarily flashes rhythmically; the female responds after a relatively fixed delay. The female-male interflash interval therefore tends to have a regular duration. However, this does not mean that the male flashes in response to the female's answer to his preceding flash. Rather, the regularity of the female-male interval is an artifact of the male's ordinarily rhythmic flashing. If he has to detour around or over obstacles, his flash may be long-delayed. Females remain responsive for many minutes without photic input.

Though dialog males do not flash in response to the female's answering flash, they may give photic responses under other circumstances. During the courtship of *Luciola lusitanica,* the male flashes about once per second, and the male-female delay interval, the key to recognition of the female by the male, is about 0.3 s (Papi, 1969); but Papi also observed instances in which the flash of a flying male triggered a flash by a male in the grass, after a delay of about 0.3 s. Males giving such "homosexual" (sic) responses remained on foot and sometimes, by answering flying males repeatedly, induced them to land. This behavior is thus unusual both in involving male-male photic interaction and because the response simulates the normal delayed response of the conspecific female. Papi recognized that ". . . to some extent [the behavior] is identical in the two sexes, indicating unsuspected common central [nervous] mechanisms."

In *Photinus concisus,* the male's flashing period is about 2 s and the female's code-key response delay is about 0.6 s (Lloyd, 1968). In this species, perching males often attract flying males by responding after a delay of 0.6 s (Buck and Buck, unpub.). Similarly, grounded males of *Photinus aquilonius* sometimes answer a flashlight signal after about the female's normal delay interval (Dr. Sara Lewis, pers. commun.).

Though males of the above-mentioned three species sometimes flash a response to other males after a delay equal to the normal male-female response delay, no con-

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Abbreviations: LED = light-emitting diode; STR = straight-ahead flight; CIR = circular flight; SD = short delay; and LD = long delay.

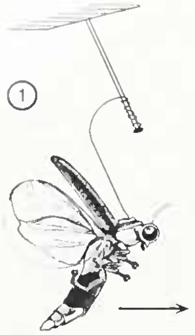


Figure 1. Lateral view of male attached to STR suspension of No. 30 chromel wire. Not to scale (actual length of insect, 15 mm; of insect-to-pin wire, about 30 mm).

sistent behavior of this sort has been described in either *Photinus pyralis* or *P. greeni* during many years of study. Flying male A of *P. pyralis* may indeed orient toward the flash of flying near-neighbor B, if B happens to flash about 2 s after one of A's flashes. However, A does not flash an answer to B, and B pays no attention to A, so the attraction breaks down.

Flying males of *P. pyralis* may also be stimulated to flash, but not orient, by the flash of another male flying nearby. This occurs during synchronized flashing (Buck, 1935) and between males flying and flashing indoors in total darkness (Buck, 1938a), but the delay involved is less than half a second. The quantitative relationships of this short-delay response to the 2-s orientational response, and to the normal 6-s flashing period, are virtually impossible to obtain from photometer measurements on a given male in the field, because of the unpredictable changes in direction, velocity, altitude, recording distance, and body orientation during flight. The relationships are also impossible to obtain from captive (perched) males because such animals rarely flash spontaneously, and never regularly.

In an effort to induce males to flash rhythmically from a fixed position that would permit controlled photic stimulation and recording, I attempted to induce flight in tethered specimens in the laboratory. As will be shown, this technique did permit recording of spontaneous flashing and of normal photic responses. Unexpectedly, it also evoked a new behavior suggesting that circuitry mediating response at the female's characteristic delay interval is present in the male in latent form.

Materials and Methods

The principal laboratory data were derived from *P. pyralis* males collected near Bethesda, Maryland, during June and early July of three seasons. Supplementary measurements were made on Baltimore males studied in Woods Hole, Massachusetts, during late July and early August of a fourth season. Field observations totalled ten years.

Netted specimens were stored in closed 10-ml plasma vials humidified with a chip of raw apple, and were used at various times over several days at 23° to 26°C. Two types of suspension, pivoting on No. 2 insect pins, were used in inducing tethered flight. On one (STR; Fig. 1) the male maintained a fixed direction. On the other (CIR; Fig. 2) the animal flew in a tight circle. The suspensions held the animals a few cm above a black benchtop.

Specimens were immobilized with carbon dioxide or on a chilled porcelain plate, and the suspension was cemented to the center of the pronotum with a droplet of low melting point dental wax, using an electrically heated needle (Buck, 1938b). After mounting, the specimen was given an 8 × 8 mm slip of filter paper, dampened with sucrose water, to hold and drink from. Rectangular flashes of 0.4 s duration, from a green light-emitting diode (LED; Monsanto MV5253), were the standard photic stimulus. These were delivered at a level slightly below the firefly, 15–20 cm from the pivot. For the STR males the stimuli were lateral. Flashes were detected at bench level with an RCA 1P21 photomultiplier photometer 25–30 cm from the pivot, and recorded on a chart recorder at 25 mm/s (Buck and Buck, 1968). Stimulus-response delays were measured on the chart from rise to rise, with a time resolution of 0.01 s.

In testing, the pivot pin was positioned in cork so as to hold the animal in correct flight attitude. Light intensity at the firefly's level was reduced to typical evening field level (5–20 lux) by replacing the room light with a single shielded 40-W S11 lamp reflected off a whitish sound-tile ceiling eight feet above the bench. (This level of illumination enables one to see head and abdominal movements.) The tarsal flight reflex was then evoked by removing the filter paper from the feet. Occasionally, flight initiation was encouraged by blowing on the insect.

Significance of differences between means was as-

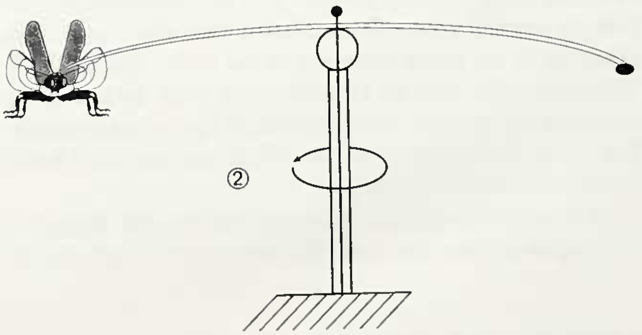


Figure 2. Facing male mounted on CIR suspension consisting of a 22 cm × 2 mm strip of thick photographic film resting on glass bead. Pronotal end of strip tapered. Other end with wax bead tare. Not to scale.

sessed by Student's *t*-test. Mean response delays are given with standard deviations (s), not standard errors. In making comparisons, variance is indicated by V, the coefficient of variation (s/M).

Results

Flight

On both suspensions, about two thirds of the hundred-odd males mounted flew reliably; about two thirds of those flying responded to LED stimulation. Flight variability was possibly due to the difficulty in mounting the animals so that the suspension did not touch the spread elytra or antennae; response variability was perhaps due to the body not being in exactly the normal flight attitude [head extended forward from under the nearly horizontal pronotum, thorax inclined downward toward the rear about 30°, abdomen hanging down almost vertically, directing the light from the ventral lanterns in the 6th and 7th segments downward and forward (Fig. 1)]. The flight reflex is very compelling; males will even fly upside-down.

With both suspensions, many continuous flights of up to 30 min were observed, and some animals flew on more than one day if demounted between flights. Wing-beat frequency of STR animals was found stroboscopically to be about 80 Hz at about 25°C. Flying STR animals sometimes writhed the abdomen, a behavior that *P. pyralis* does not exhibit in normal flight. STR males that flashed spontaneously (*i.e.*, more than 4 s after an LED flash; see Discussion) showed no flight change corresponding to the dip and hover behaviors that are normally associated with flashing during field display. When given an LED answer about 2 s after flashing, males sometimes turned the head immediately toward the signal (video observations with J. F. Case). Presumably this was the functional equivalent of the normal response in which the body turns as a whole.

Many CIR records showed an artifact due to ambient light reflected off or interrupted by the rotating arm (Figs. 7a, 16, arrows). From the typical, indicated 2-Hz rate of rotation, and the 11-cm radius of rotation, the velocity of linear flight was calculated to be about 3 mph. Rate of rotation was constant.

The results reported below suggest that a normally hidden 2-s flash-control circuit of the *P. pyralis* male was sometimes revealed by forcing the animal to fly in a circle. As a control background for this hypothesis, I therefore first present full ranges of both the spontaneous flashing and the short-delay photic responses of both STR and CIR males.

Spontaneous flashing

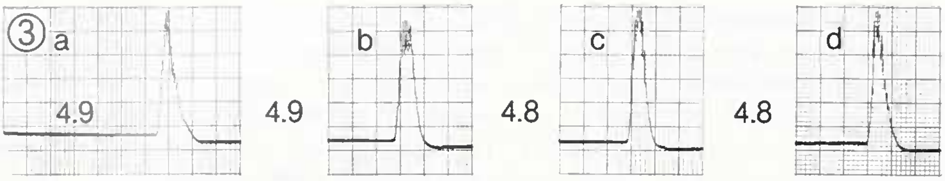
Flashing in the absence of stimulation was usually sporadic, but, on both suspensions, some males flashed spontaneously and consecutively for a number of cycles in approximately the normal rhythm. These spontaneous flashes were sometimes quite uniform in intensity and were emitted as regularly as by males flashing in the field. The STR series of Figure 3, for example, shows uniform flashes and regular rhythm. In contrast, the Figure 5 STR series illustrates flashes that were highly variable in form (perhaps because of abdominal twisting), though emitted with respectable regularity (see figure legends). With the CIR suspension, apparent flash intensity, contour, and duration varied widely as the animal rotated and the ventral abdominal lantern was alternately partly occluded and then exposed to the photometer, but rhythmic flashing was nonetheless observed (Fig. 7 and legend).

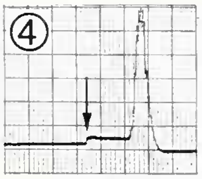
Short-delay photic response (SD)

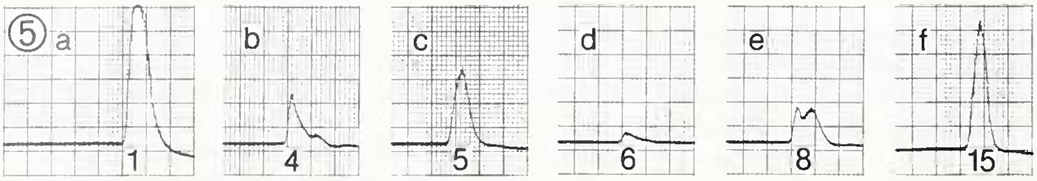
The effects of exposing flying males to LED flashes were quite variable in mode, between individuals, and between suspensions, and often the firefly did not flash for many seconds after a signal. Two types of consistent response were observed. In the more frequent, seen in animals on both suspensions, the male flashed from 0.2 to 0.7 s after the LED stimulus. Many hundreds of these "triggerings" were recorded from the dozens of males studied. Figures 4 and 9–14 illustrate variations in flash form and delay observed in six STR males, and Figures 8 and 16–18 do the same for two CIR animals. Mean response delays for individual males ranged from 0.26 ± 0.02 s to 0.46 ± 0.08 s, and some differed significantly from each other. Mean delay ranges for individual males were typically less than 0.15 s.

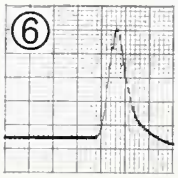
CIR animals tended to respond after somewhat longer delays, and flash more dimly, than STR animals, although there were substantial overlaps. I found that males walking on a smooth horizontal surface did not respond to flashed answers to their signals if the answer was delivered from directly behind or from the rear within 30° to either side of the longitudinal body axis. This means that CIR males might have been unable to see the LED signals for up to 1/6 revolution (*ca.* 0.083 s).

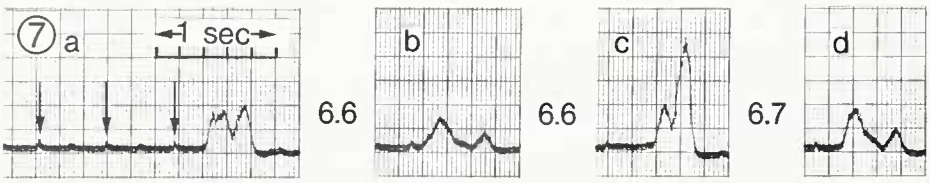
When apparent CIR delays were each reduced by an average blind-spot correction (0.04 s), the overall mean delay duration was not significantly greater than that of STR animals at the same temperature. Ninety-five percent of both STR and CIR measurements fell between 0.25 and 0.65 s. For the present, accordingly, the most parsimonious conclusion is that STR and CIR males give the same short-delay photic response. The narrow frequency distribution peaking at 0.4 s in Figure 19 is the averaged delay for STR and CIR males. The mean delay for the combined group was 0.38 s.

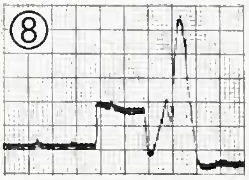


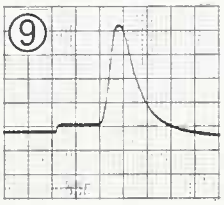


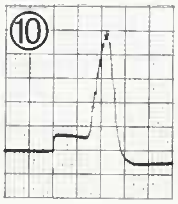


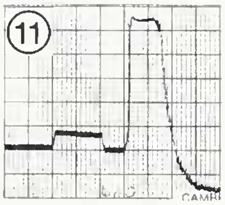


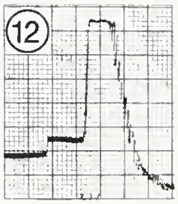


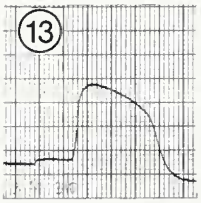


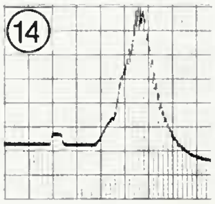


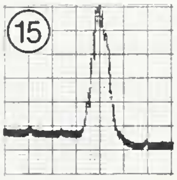


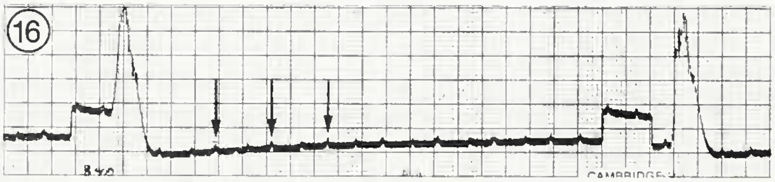


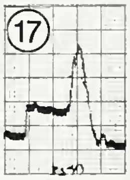












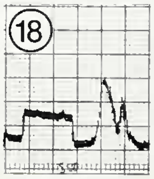


Figure 3. Four successive spontaneous flashes of STR male 126. a–d, flashes 2–5 in a rhythmic series of 8 (mean period 4.92 ± 0.16 s; V = 3). Numbers are flash-to-flash intervals. T = 25°. Note: in the chart records, the rapidly rising and falling limbs of some flashes have been reinforced. Jagged or sawtooth traces are instrumental AC noise revealed by high amplification. Decline of traces below baseline after some flashes is an instrumental artifact. Flash intensity is arbitrary. Time scale for all records indicated on Figure 7a. **Figure 4.** SD response of STR male 126 to 0.4 s LED stimulus. Delay 0.35 s, showing stimulation at the LED "on" phase (arrow). **Figure 5.** Rhythmic spontaneous flashing of STR male 124. a–f, flashes 1, 4, 5, 6, 8, and 15 in a series of 27, showing variability of flash intensity and form. Record also showed a 0.4-s difference in mean delay between two sections of same run; mean of first 13 cycles, 6.06 ± 0.41 s (V = 6); mean of last 14 cycles, 6.46 ± 1.04 s (V = 16). **Figure 6.** Spontaneous flash of STR male 132. T = 25°. **Figure 7.** Four consecutive spontaneous flashes of CIR male 94. a–d, flashes 9–12 in rhythmic series of 13, showing 0.55 s rotation artifacts (arrows in a) and effects of rotation on flash form delineation by photometer. Numbers, interflash intervals. Mean period for series, 7.2 ± 0.93 (V = 13). T = 22°. **Figure 8.** SD response of CIR male 94. Delay 0.46 s. **Figure 9.** SD response of STR male 1. Delay 0.36 s. **Figure 10.** SD response of STR male 98. Delay 0.28 s. **Figure 11.** First of two consecutive SD responses of STR male 105 that were 5 s apart. Peak clipped by over-amplification. Delay 0.60 s. **Figure 12.** Next SD response of STR male 105. Delay 0.3 s. **Figure 13.** SD response of STR male 282, showing 0.8 s flash duration. Delay 0.3 s. T = 27°. **Figure 14.** SD response of STR male 123 to 0.1 s LED flash, showing slow light accretion but typical 0.37 s delay. **Figure 15.** Spontaneous flash of CIR male 107. T = 23°. **Figure 16.** Two consecutive SD responses of CIR male 107, 4.4 s apart. First delay, 0.37 s; second delay, 0.58 s. Arrows, rotation artifact. **Figure 17.** SD response of CIR male 107 showing delay of 0.35 s, close to that of first flash in Figure 16, but of lower intensity. **Figure 18.** SD response of CIR male 107, showing delay of 0.56 s, close to that of second flash in Figure 16, but of lower intensity, and distorted by rotation of suspension.

Long-delay photic response (LD)

A second, less frequent type of photic response (Figs. 20, 22–24; filled columns in the 1.5 to 3 s range in Fig. 19) was given only by CIR males. In 15 of 31 individuals, from 1 to 23 such responses, with delays averaging 2.3 s, were recorded, sometimes several in succession. No flashes in the 2–3 s post-stimulus range were seen in any of 20 different males flying from the STR suspension. In some instances, the CIR firefly's response flash was followed by a second, spontaneous flash at about the same interval (Fig. 24). On several occasions, two spontaneous flashes about 2.3 s apart were emitted (Figs. 21, 24; unfilled column caps in Fig. 19).

In sum, LED stimuli evoked two photic responses: a short-delay (SD) variety—delayed an average of 0.38 s (Fig. 19, first distribution frequency peak); and a long-delay (LD) one—delayed an average of 2.3 s (Fig. 19, 1.5 to 3 s concentration). Both distributions were shown to be significant ($P > 95\%$) by Wallenstein's (1980) scan statistic, using an 0.5-s window. With one exception among hundreds of records (Fig. 23), LED stimulation evoked one response or the other, not both.

Discussion

Spontaneous flashing

In experiments on intact fireflies, there is no direct way of ascertaining which flashes are initiated endogenously and which are responsive. In the present work, absence of significant clumping of flashes later than 3 s after LED stimulation (Fig. 19) indicates that the SD and LD distribution peaks reflect true photic responses, and conversely that firefly flashes that occurred more than 3 s after exogenous input were spontaneous (endogenous). This conclusion is supported by both field and laboratory observations. Of 378 display-flashing periods recorded in 18 series, each from a different male of *Photinus pyralis* flying free in the field at 23° (ave. 6.15 s), only one was shorter than 4 s.[2] Similar results were obtained by Edmunds (1963) and Maurer (1968).

Though spontaneous STR and CIR flashing tended to be less regular than in the field, due to frequent flash-skipping, series of consecutive flashes were well within the reported range of field rhythm variability[2] (legends of Figs. 3, 5, and 7). The special case of 2–3-s pairs of apparently spontaneous flashes (Fig. 21; unfilled column caps in Fig. 19), is discussed below.

The SD ("reflex"?) response

Aside from irregularities in recorded flash form due to the motion of the CIR males, the SD photic responses observed in STR and CIR males differ in no essential respect from each other, or from those seen normally in free-flying animals.[3]

Short-delay photic interactions between males of *P. pyralis* had been observed in synchronized flashing among males courting the same female (Buck, 1935; Maurer, 1968) and between males flying indoors in darkness (Buck, 1938b), but it was not until the response was measured and studied intensively that it was recognized as part of the male's normal repertory (Buck *et al.*, unpub.). By programmed stimulation, the triggering was confined to the latter half of the flashing cycle (*i.e.*, more than 3 s after a male's flash)—an interval dubbed the "late window" to distinguish it from the 1.5–2.5-s post-flash "early window," which is tuned to the female's response and mediates orientation (Case, 1984; Buck, 1988).

In the field, the SD interaction occurs as a triggering of one flying male by the flash of a *close* neighbor or of an artificial light. The present laboratory-triggered delays are consistent with those measured in video records from free flying males (Buck *et al.*, unpub.). When male A thus triggers the flash of B, neither animal pays any attention to the other, but B's flashing rhythm is reset so that thereafter he flashes in synchrony with A (Case, 1984; Buck,

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[2] The many hundred raw measurements in Buck (1936) that were given antique statistical treatment by Buck (1937) were reexamined to assess the range of individual period variation in free-flying males in nature. Using 38 series of 8 to 71 consecutive flashes, it was found: that a typical V value is 10, with values as low as 3 and as high as 20 occurring occasionally; that mean period decreases about 0.4 s for each degree (C) rise in temperature; and that statistically significantly different individual means occasionally occur even between two individuals at the same temperature. Nonetheless, as shown by Buck and Buck (1968, footnotes 42–47), the *P. pyralis* rhythm, though inferior in regularity to those of some tropical synchronizing species, is quite in line with many other biological periodicities, including human heartbeat during sleep.

[3] The variations were not necessarily due to the experimental conditions. Even in free field flashing, the magnitudes of all flash parameters show centrally peaked frequency distributions, and all vary with temperature. In considering photic effects on flash *timing*, flash initiation (with which this study is principally concerned) must be distinguished from flash *modulation*. Initial excitation depends on neuronal volleys from the brain (Case and Buck, 1963; Buonamici and Magni, 1967; Brunelli *et al.*, 1977) but there is some evidence that flash form (intensity, duration, time-course) may be affected also by activity of the final cord ganglia (Christensen and Carlson, 1981). The number, firing sequence, and areal distribution of the individual flashing units may also vary (Buck, 1955, 1966; Hanson *et al.*, 1969). Thus there are many potential sources of variation. Some STR records show flash form varying independently of the spontaneous flashing rhythm (Fig. 5), and between individuals given comparable stimulus flashes (Figs. 9–12). Aside from effects of changing firefly-photometer geometry, the same conclusions hold for the flashes of CIR males (Figs. 7, 8, 15–18, 20–24). The important point is that both the SD and LD photic responses maintain their characteristic and exclusive *delay* ranges independently of variations in flash *form* between individuals and between runs.

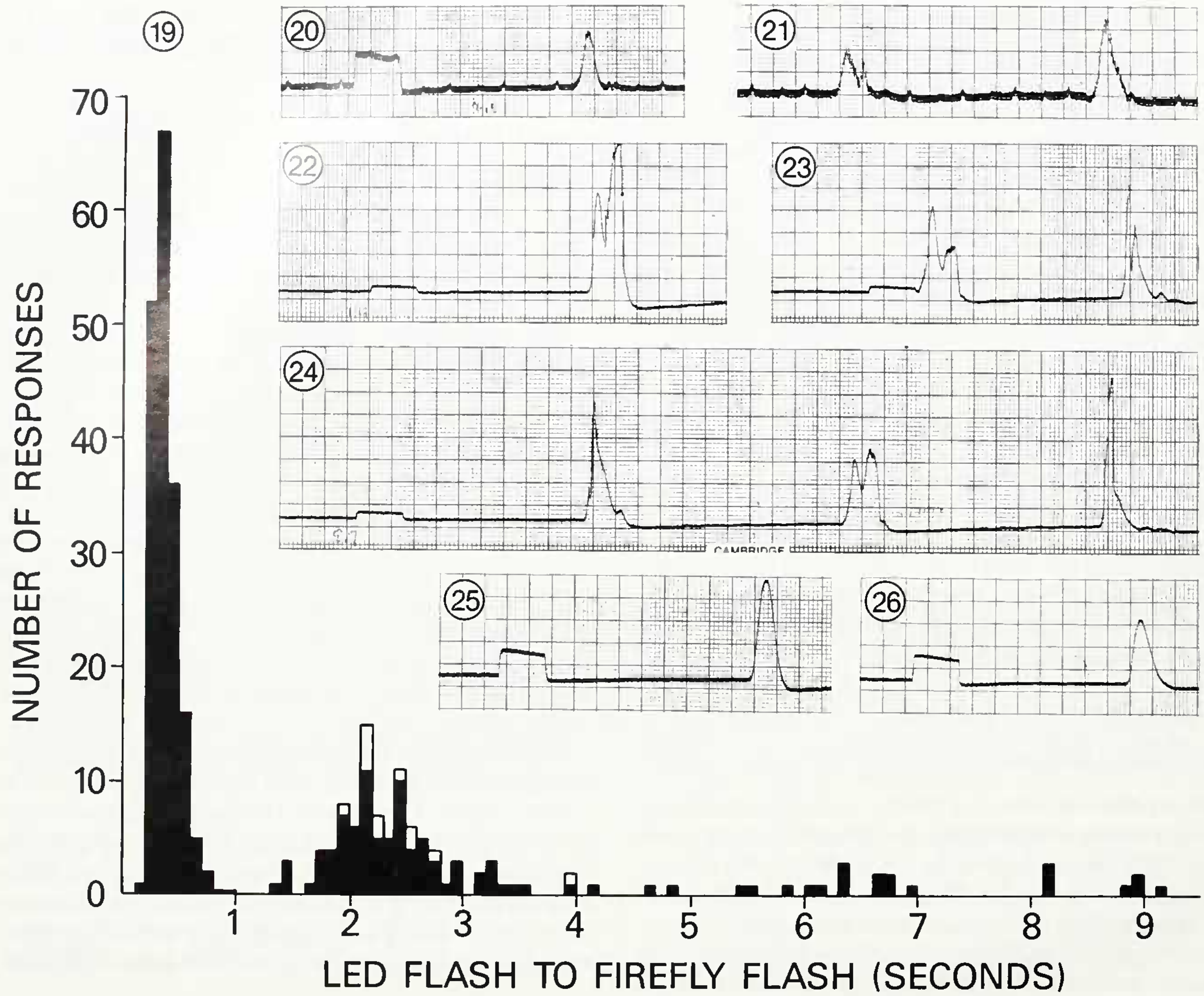

Figure 19. Frequency distributions of SD and LD responses. First peak (0.2–0.8 s) is the average of 180 SL responses of 6 STR males and 180 SD response of 6 CIR males (30 consecutive responses for each individual). Second peak (1.3–3 s) is 72 LD responses of the 10 CIR males that emitted more than one LD flash (filled columns), plus 11 corresponding spontaneous flash-to-flash intervals from the same males (unfilled caps). **Figure 20.** LD response of CIR male 107. Delay 1.96 s. **Figure 21.** Pair of spontaneous flashes 2.2 s apart. First flash was 11 s after previous flash. CIR male 107. **Figure 22.** LD response of CIR male 250. Delay 1.94 s. T = 22°. **Figure 23.** Rare apparent SD and LD responses to same LED flash. SD delay 0.44 s; LD delay 2.2 s. CIR male 250. **Figure 24.** LD response (delay 2.0 s), followed by two spontaneous flashes, the first 2.3 s later, the second 2.3 s after the preceding. CIR male 250. **Figure 25.** Normal response of freely perched female to LED flash. Delay 2.3 s. Female 1. **Figure 26.** Same as Figure 25. Delay 1.92 s. Female 2.

1988). The figures in the present paper are intended only to illustrate the variation range of the response. Its detailed aspects will be taken up in another paper. Its putative functions are discussed by Buck (1988).

The SD male-male triggering behavior in *P. pyralis* is also of interest because its delay is often not greatly different from that for flashes elicited by electrical stimulation in the head (Case and Buck, 1963). Similarly, the 0.3-s delay of the *L. lusitanica* male-male response (Papi, 1969) corresponds to the electrical brain delay in that species (Brunelli *et al.*, 1977). A 0.3-s photic response, distinct from the 0.6 male-female interval, has been found also in *P. concisus* and shown to correspond to the head-lantern electrical delay in that species (Hanson and Buck, unpub.). [It may also be the inter-male interval involved in the synchronized field flashing observed by Otte and Smiley (1977).]

Papi used the term "reflex" for the SD male-male interaction interval in *L. lusitanica*, perhaps implying that it involves the sort of minimum brain-lantern delay expected in a nonspecific reflex—that is, a fixed response inherent in the way the flash-control system is constructed rather than one evolved specifically in a communicative context. (The human knee-jerk, a response incidental to the presence of stretch-receptors that function normally in locomotion, is a case in point.) In this vein, and because of the lack of interaction between free *P. pyralis* males after such triggering, I use "reflex" provisionally to suggest a possible qualitative distinction between the 0.38-s SD and 2.3-s LD photic responses.

The LD response (female-type circuit in male)

Because males of *L. lusitanica, P. concisus,* and *P. pyralis* respond (by orienting) to the characteristically delayed response flashes of their conspecific females, each must have a response-timing circuit that corresponds to the emission-timing circuit of the female. Males of *L. lusitanica* and *P. concisus* also flash in response to other, conspecific males, and after the same delay used by their respective females. This suggests that the timing process initiated by seeing a flash of light may, potentially, terminate by mediating either orientation or flashing. Whether this lability involves bifunctional or parallel circuits, and what determines whether female simulation occurs normally (*L. lusitanica, P. concisus*) or not (*P. pyralis*), are less important in the present context than the apparent presence in both sexes of the same emission-timing.

The 2.3-s (LD) signal-male delay induced in CIR *P. pyralis* males (23°) should presumably be shortened about 8% as a rotational correction, but is, in any case, close to the average 2.1-s response delay of *P. pyralis* females answering flashlight flashes at about 23° (Buck, 1937) or LED signals (Figs. 25 and 26). Thus, this laboratory finding appears to parallel Papi's SD finding and to strengthen the idea that emission-timing circuitry of the female type is also present in the male.

Why female-simulating behavior is overt in *L. lusitanica* and *P. concisus* and latent in *P. pyralis* is unknown. *P. pyralis* is the most abundant and widespread American photinid firefly, occurring in at least 23 states (Lloyd, 1966), whereas its sibling species, *P. concisus*, is limited to a small area of central Texas. These distributions are consistent with the expectation that a signal that identifies the female unambiguously would have selective advantage over one that does not. Possibly *P. pyralis* has evolved a step beyond *P. concisus*.

There were not enough spontaneous intervals of 2–3 s duration (Figs. 21, 24; unfilled column caps in Fig. 19) to assert that they derive from endogenous excitation of the same LD flash-timing circuit that is sometimes excited by LED flashes (Figs. 20, 22, 24). However, the concentration of such intervals strongly suggests that the stress of flying on the CIR suspension does induce spontaneous flashing at 2–3-s intervals in addition to the also atypical 2.3-s LD photic response to LED stimulation.

Use of male circuitry by female

In the three fireflies discussed above, the male recognizes the female's emission pattern specifically, but there is no evidence that the female recognizes the rhythmic spontaneous interflash interval of the male. However, in certain species in which the male's emission signal is a pair of flashes rather than a single flash, the female does recognize the male specifically. She responds only after being presented with a pair of flashes timed in the characteristic pattern of her conspecific male, and thus must have a circuit tuned to that interval. In *P. greeni*, for example, the male emits a pair of flashes 1.5 s apart every 5 or 6 s, and the female responds about 0.8 s after the *second* flash of the 1.5-s pair (Lloyd, 1969; Buck and Buck, 1972).

No instance has been reported of a female of a pair-flashing species mimicking her male's flash pattern in the field, but *P. greeni* females have been induced to flash in pairs 1.5 s apart by strong repetitive photic stimulation (Buck and Case, 1986). Thus, as with *P. pyralis* males forced to fly in tight circles, it appears that abnormal stimulation sometimes uncovers latent flash-timing capacity.

Significance and genesis of unisex flash-controls

In a cricket in which females cannot call, Huber (1962) found that females nevertheless ". . . possessed a nervous organization sufficient for primitive stridulatory movements in spite of the absence of stridulatory structures." Alexander (1962) suggested that if both sexes were at least potentially able to call ". . . it would represent an interesting simplification of evolutionary change in a communicative system—something of an assurance that the . . . song of the male and the ability of the female to respond to it . . . will evolve as a unit." The present evidence that male and female dialog fireflies share specific, quantitatively matched, flash-timing controls, overt or latent, may implement Alexander's insight.

Because males and females of dialog fireflies are almost identical in lantern structure and control mechanisms (Buck, 1948), the shortest photic delay circuit, if it is indeed a reflex, would be expected to be present in both sexes. It would be understandable, then, that this circuit could have been co-opted during evolution to mediate both the female's response delay and the matching recognition interval in the male (*L. lusitanica* and *P. concisus*), and to confer supplementary reproductive advantage via male flash synchronization (Buck, 1988) in *P. pyralis* and *P. concisus.*

It is less obvious how and why, in some species, this potentially unerring clue to female identification has evolved (or retained) the ambiguity of being used by males as well as females. The surmise that dialog questions and answers ought to evolve as a unit seems not readily compatible with paradoxical behaviors like those in *L. lusitanica* and *P. concisus* in which males normally decoy other males as well as seek females. The existence of female-signal simulation seems to argue that the duplicate timing circuits owe their evolutionary fixation to that behavior, but it also seems obvious that dialog in which males can identify females unequivocally (as in *P. pyralis*) should be more strongly selected than dialog in which males are also attracted by males.

Among suggested functions of female-simulation, "improving the female's chances of fertilization" (Papi, 1969) implies altruistic group selection. "Giving a rejected male an opportunity to see and approach the female's flashed answers to another male, and thus another chance to mate with her . . ." (Lloyd, 1979) seemingly has the rejectee and the female synchronizing with each other as both flash in response to the primary male. This would require the rejectee to recognize a flash that not only did not occur at the proper female-recognition interval after his flash but was in a phase relation (simultaneity) that has been found, in other species, to be the point of minimum sensitivity to photic input (Buck *et al.*, 1981; Buck, 1988). It is also not at all clear that males giving the female-simulating response have, in fact, been rejected previously.

A third possible function of female-signal simulation—distracting the deceived male from courting the real female, and so boosting the decoy's statistical chances of finding a mate (E. Arbas and S. Lewis, pers. comm.)—may have more promise, particularly, as Dr. Lewis has pointed out to me, with the strongly male-biased operational sex ratio that is usual in dialog populations early in the season.

Summary

1. In timing her flashed answer to the male's signal, a female dialog firefly uses the same delay interval that the male uses in timing the interval between his own flash and her answer.
2. In three species, males answer the flashes of other males after the same specific response-delay interval that is characteristic of their conspecific females.
3. Experimentally, the male of a fourth species has been shown to be capable of flashing responsively after the same delay interval as the female. In a fifth species, the female can be induced to emit flashes with the same timing as one element of the male's spontaneous display.
4. The above data are compatible with the hypotheses that male and female firefly share some of the same courtship flash-timing circuits in overt or latent forms, and that a particular control circuit may, on occasion, time either detection or emission. The overall neurophysiological picture is of a pool of timing circuits that can connect in various input/output combinations to mediate a variety of behavioral patterns.

The data are consistent with Alexander's (1962) surmise that courtship questions and answers should evolve together. All present-day circuitry must, of course, derive by selection from ancestral flash-controls. In another communication I plan to compare firefly unisex responses with possible analogs in other animals, and to examine the speculation that duplicate circuits in conspecific male and female fireflies hark back to a stage in dialog evolution in which both sexes flashed alike.

Acknowledgments

Many colleagues have enhanced my understanding of control physiology and evolution theory, but Drs. Edmund Arbas, Albert Carlson, Joseph Cicero, Sara Lewis, and Stephen Shaw helped particularly with the present communication. Jon Copeland suggested trying tethered flight. Richard Raubertas recommended the Wallenstein statistical analysis. I am also grateful to Elisabeth Buck, James Case, and Frank Hanson (the "*et al.*" cited in the text) for allowing reference to unpublished joint work, to Dr. Hanson for firefly shipments, and to Jeff Carpenter for field assistance. Charlette Lancaster made the drawings.

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The Structure of Sweeper Tentacles in the Black Coral *Antipathes fiordensis*

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Abstract. Normal tentacles on polyps of the black coral *Antipathes fiordensis* are less than 2 mm long and display well-defined, wart-like structures, the centers of which are marked by both flagella and microvilli. Both of these microappendages are characteristic of spirocytes, the dominant type of cnidocyte in normal tentacles. Sweeper tentacles, up to 15 mm long, form in apparent response to an alcyonacean epibiont. The external surface of the sweeper tentacle lacks the well-defined, wart-like batteries of the normal tentacle, and exhibits a general reduction in the appearance of surface microappendages. Nonetheless, there is a greater number of cnidae per unit area. No spirocysts are found in these sweeper tentacles. Instead, the cnidom is composed entirely of microbasic b-mastigophores (MbMs). More than 99% of these are of a single type that are structurally different from the MbMs found as a minority of the cnidae in normal tentacles. Changes in sweeper tentacle cnidae are compared with those occurring in modified tentacles of other anthozoans.

Introduction

Aggressive behavior by anthozoan coelenterates is effected by a variety of specialized structures containing nematocysts (see review by Bigger, 1988). Extrusion of septal filaments (preferred to "mesenterial" filaments; see Bayer and Owre, 1968) onto a competitor is a common strategy among scleractinian corals (Lang, 1973; Glynn, 1974; Loya, 1976; Cope 1981; Bak *et al.*, 1982; Logan, 1984), and has been described in a corallimorpharian as well (Chadwick, 1987). Some anemones possess specialized marginal vesicles—the acrorhagi—that are used for this purpose (Francis, 1973; Ottaway, 1978; Bigger, 1980; Ayre, 1982; Sebens, 1984). A few anthozoans are capable of forming specialized tentacles during aggressive interaction. den Hartog (1977) described the formation of specialized bulbous tentacle tips in corallimorphs after contact with stony coral competitors. In other species, the entire tentacle structure can be modified under such conditions. Some acontiate anemones develop short, opaque, blunt-tipped "catch" tentacles (*e.g.*, Williams, 1975), while some scleractinian corals can form elongated "sweeper" tentacles (Lewis and Price, 1975; Bak and Elgerschuizen, 1976; den Hartog, 1977; Richardson *et al.*, 1979; Wellington, 1980; Chornesky, 1983). Sweeper tentacles have also been described in a few gorgonian corals (Sebens and Miles, 1989 and references therein). In all these cases, the transformation occurs within 4–9 weeks, when the normal or feeding tentacle converts to a sweeper or catch tentacle as the result of contact with other coelenterate species (Wellington, 1980; Bak *et al.*, 1982; Bigger, 1982; Chornesky, 1983). Intraspecific competition (Purcell and Kitting, 1982; Kaplan, 1983; Hidaka and Yamazato, 1984; Hidaka, 1985; Fukui, 1986) and competition with non-coelenterates (Hidaka and Miyazaki, 1984) also appear to be associated with changes in tentacular morphology.

The induction of sweeper and catch tentacles is accompanied by changes in the relative proportions of nematocysts and spirocysts, as well as by shifts in the type of nematocyst (den Hartog, 1977; Purcell, 1977; Wellington, 1980; Watson and Mariscal, 1983a, b; Hidaka and Yamazato, 1984; Fukui, 1986; Hidaka *et al.*, 1987). However, these earlier observations were made with optical or scanning electron microscopes and the intracap-

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sular details of cnidae before and after conversion to the sweeper tentacle were not compared. Thus, only changes between classes of nematocysts could be detected, while more subtle changes within-class were not. Similarly, though some of the external differences between tentacle types have been documented (den Hartog, 1977; Hidaka and Miyazaki, 1984; Fukui, 1986; Sebens and Miles, 1989), little attention has been given to histological and cytological distinctions. Watson and Mariscal (1983a, b) examined catch tentacles from this perspective, while Doumenc (1972) and Bigger (1982) documented the ultrastructural differences between acrorhagi and tentacles. We know of no corresponding work for sweeper tentacles.

Antipatharian corals are little known, largely due to their typical occurrence in deeper water. However, on the southwest coast of New Zealand's south island, large, virtually monotypic black coral populations occur in depths of 5–30 m (Grange *et al.*, 1981; Grange, 1985), and are thereby accessible to study. The structure of the polyp, including the tentacle and gastrodermis, has been examined by Goldberg and Taylor (1989a, b) who referred to this species as *Antipathes aperta* Totton. However, further work has resulted in its re-description as the endemic *A. fiordensis* Grange, 1990. We now report on the occurrence of sweeper tentacles in these animals, and compare their morphological and cellular structure with unmodified tentacles.

Materials and Methods

The site for this study was Doubtful Sound, Fiordland, New Zealand (45° 20.95′ S, 167° 02.83′ E). Living material was photographed *in situ* at 10–20 m to document the incidence of sweeper tentacles. Color transparencies were examined microscopically to estimate tentacular size.

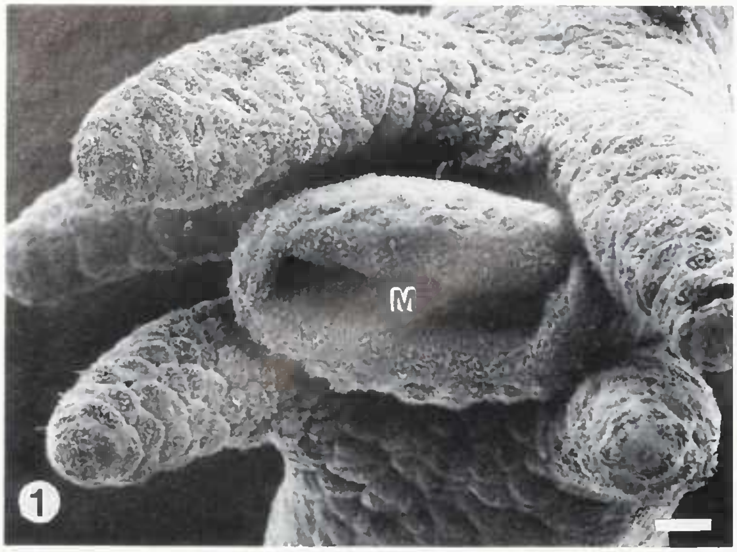
Branchlets with normal polyps and tentacles, as well as areas of the colony where sweeper tentacles had formed, were collected and immediately fixed for 4 h in a solution of 3% glutaraldehyde and 1% paraformaldehyde in seawater containing 0.1 *M* cacodylate at pH 7.4. The fixed tissues were then transferred to cacodylate-buffered seawater and express-shipped to Miami for secondary fixation in osmium tetroxide, alcohol dehydration, and embedment in Spurr resin.

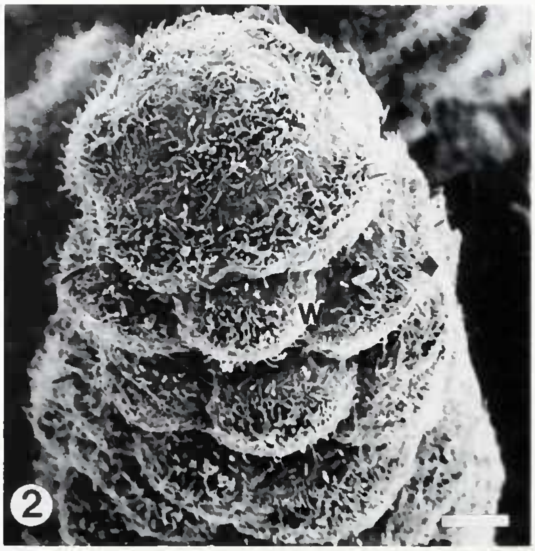
Sections of tentacle 1 μm thick were normally stained for 30 s at 55°C with borax-buffered 0.05% Toluidine blue, but sweeper tentacles required much shorter staining times (see Results). A Philips EM 200 operated at 60 kV was used for transmission electron microscopy; scanning electron microscope observations were made with an ISI Super 3A SEM, using material critical-point dried from liquid CO_2. Proportional counts of cnidae types were made by examining randomly chosen 1 μm sections taken from 8 normal and 8 sweeper tentacles until at least 1000 cnidae were counted.

Results

The appearance of the normal polyp of *A. fiordensis* is shown in Figure 1. Each polyp has six tentacles (five are shown) that surround the mouth and pharynx. The pharyngeal region also projects from the polyp as an oral cone (see Goldberg and Taylor, 1989a, for details). The living tentacles are 1.2 ± 0.6 mm long and taper to a blunt tip. There is no distinct acrosphere. The tentacle surface is organized into a series of raised, wart-like batteries, with flagella projecting from their centers (Figs. 2, 3). The warts in the apical regions are flagellated, but are better defined proximally. Ciliary cones (stereocilia surrounding a longer central cilium) are scattered and are not a prominent feature of the tentacular epidermis. Spirocysts comprise 88.7% of the tentacular cnidae. Their capsules are clear and do not stain with Toluidine blue (Fig. 3 inset). The hirsute appearance of the wart center is largely a function of the mature spirocyte (Fig. 3), the apical end of which bears a circlet of microvilli and a single, acentrically placed flagellum 10 μm or more in length (Goldberg and Taylor, 1989a).

Cells at the wart periphery and base tend to stain deeply with Toluidine blue. Part of this pattern is accounted for by the staining of the mucus cells, and part by the staining of nematocysts. Mucus cells stain a metachromatic pink, especially around the vesicles. The vesicles themselves develop a much lighter metachromasia. Most of the nematocysts stain a deep purple. The capsules average 2.5 μm wide by 18.0 μm long, and contain a triply pleated tubule in a poorly infiltrated, generally electron-opaque matrix (Fig. 3). The shaft is cylindrical, nearly the length of the capsule, and 0.5 ± 0.2 μm wide. It is approximately uniform in diameter, tapers toward the base as it meets the tubule, and ends apically in a distinctive cap. These characters are consistent with the description of microbasic b-mastigophores (MbMs) given by Mariscal (1974), and are the nematocysts referred to by Goldberg and Taylor (1989a) as type A MbMs. They comprise about 8.7% of the tentacular cnidae. A second type is larger (up to 35 μm long and 10 μm wide), and displays a weak orthochromasia with Toluidine blue. The matrix has a granular appearance in the electron microscope. Conversely, the shaft, and to some extent the tubule, are electron-opaque. The shaft is 1.8 ± 0.3 μm wide and about a third the capsule length. Despite these distinctions, this second type falls into the same nematocyst category as those described above, referred to as type B MbMs by Goldberg and Taylor (1989a). These large, granular nematocysts comprise





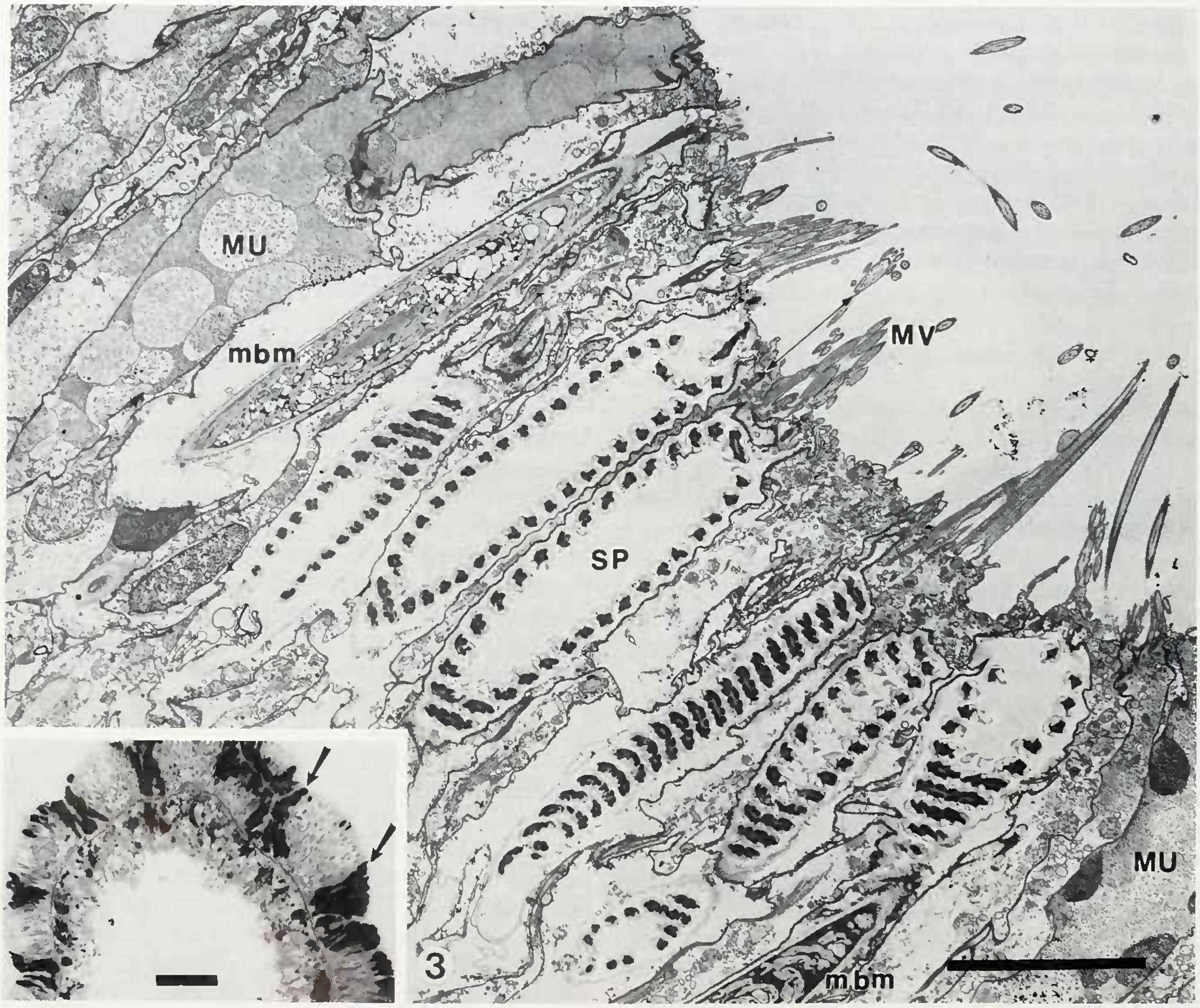
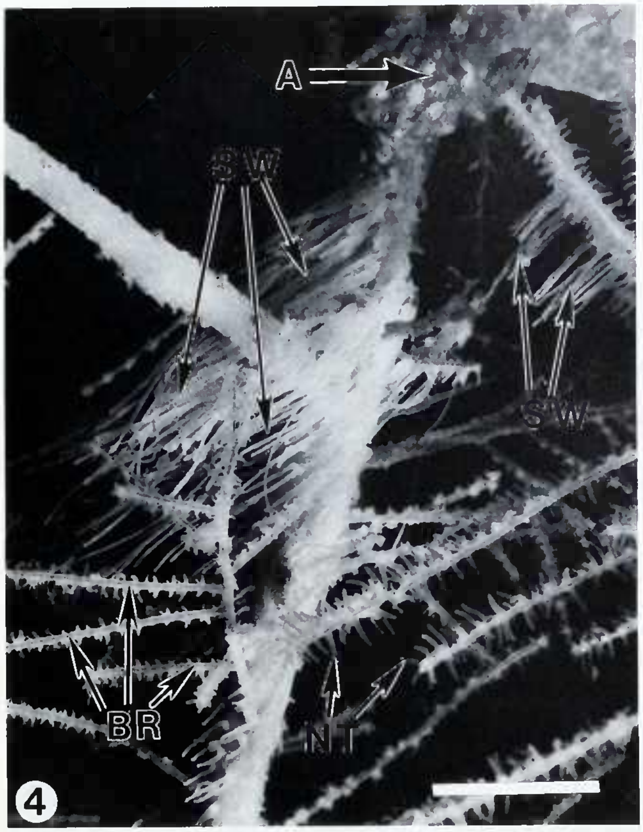


Figure 1. SEM preparation of *Antipathes fiordensis* polyp. Five of six tentacles are shown surrounding the mouth (M) in a central oral cone. Scale bar = 20 μm. **Figure 2.** Normal tentacle showing wart-like structures (W). The rim of the wart is composed of mucus cells; the depressed wart centers contain clusters



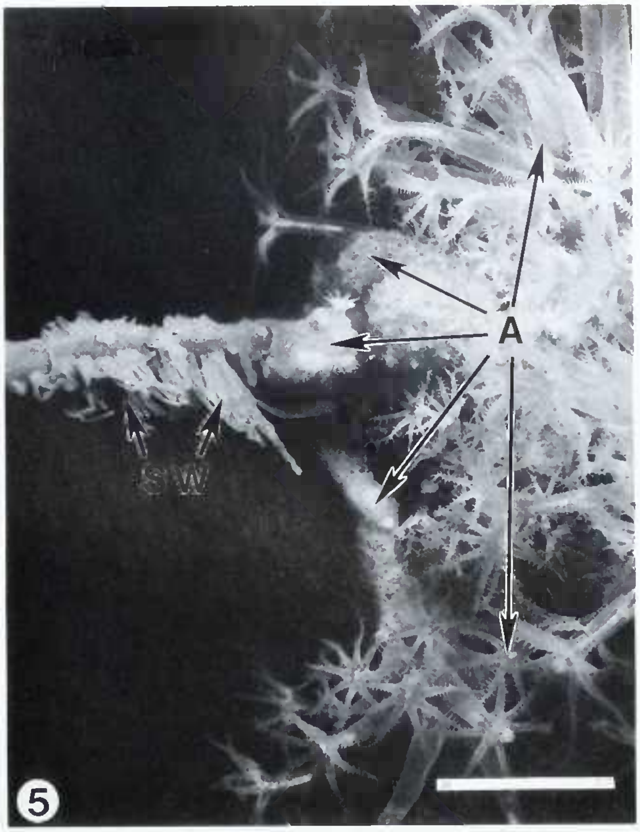


Figure 4. Sweeper tentacles (SW) adjacent to a contracted alcyonacean colony (A). Polyps with normal tentacles (NT) are located on branchlets (BR), while sweeper tentacles are usually confined to larger branches. Scale bar = 5 mm. **Figure 5.** *In situ* photograph showing development of sweeper tentacles (SW) in association with expanded polyps of the alcyonacean coral *Alcyonium aurantiacum* (A). Note apparent gradation of tentacular length toward the epibiont. There are no normal *A. fiordensis* tentacles in this photograph. Scale bar = 5 mm.

about 2.6% of the cnidae in the normal tentacle, but are the dominant cnida in the gastrodermis (Goldberg and Taylor, 1989b).

Sweeper tentacles in *A. fiordensis* are thread-like and extend up to 15 mm long (Fig. 4). A gradation of tentacle length may occur, either through contraction, distance from the competitor, or both, so that some tentacles may be as short as 2 mm. We have examined all tentacle sizes histologically (see below), and have not found intermediates between normal and sweeper tentacles. There is no distinct acrosphere, in contrast to their formation or enlargement in other corals with sweeper tentacles (den Hartog, 1977; Chornesky and Williams, 1983; Hidaka and Miyazaki, 1984; Sebens and Miles, 1989), and their agonistic analogues in corallimorphs (den Hartog, 1977). *A. fiordensis* sweeper tentacles tend to form on thicker (older) branches, generally 4 mm or more in diameter and are especially common in the presence of the alcyonacean *Alcyonium aurantiacum* Quoy and Gaimard (Fig. 5). We have also noted them near red algae (*Epymenia* sp. and *Lithothamnion* sp.). However, in these instances we have not ruled out the presence of foreign

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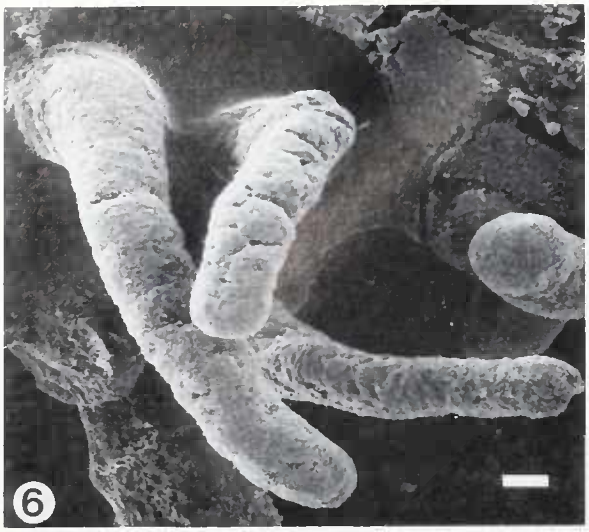
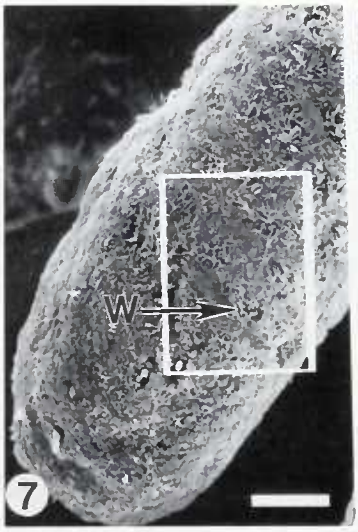
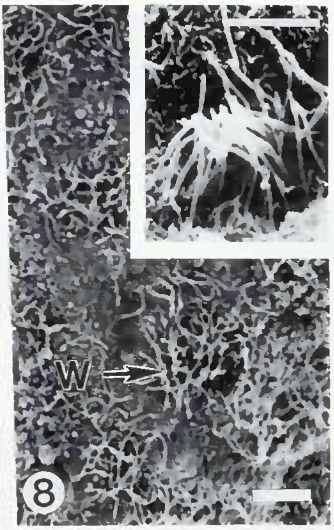
of flagella, representing the apical ends of cnidocytes. Scale bar = 20 μm. **Figure 3.** TEM preparation through a wart center perpendicular to the tentacular axis. Flagella and prominent microvilli (MV) are associated with the surface of mature spirocytes (SP). An arrow connects the flagellum and rootlet in this spirocyte. Two type A microbasic b-mastigophores (mbm) are shown. Upper left: a mature capsule with characteristic poorly infiltrated matrix. Lower right: an immature capsule with typical electron-opaque matrix. The raised edges of the wart are formed by mucus cells (MU). Scale bar = 5 μm. Inset: tentacular cross-section showing arrangement of stained mucus cells (arrows) surrounding unstained spirocyte clusters. Light microscopy; Scale bar = 20 μm.







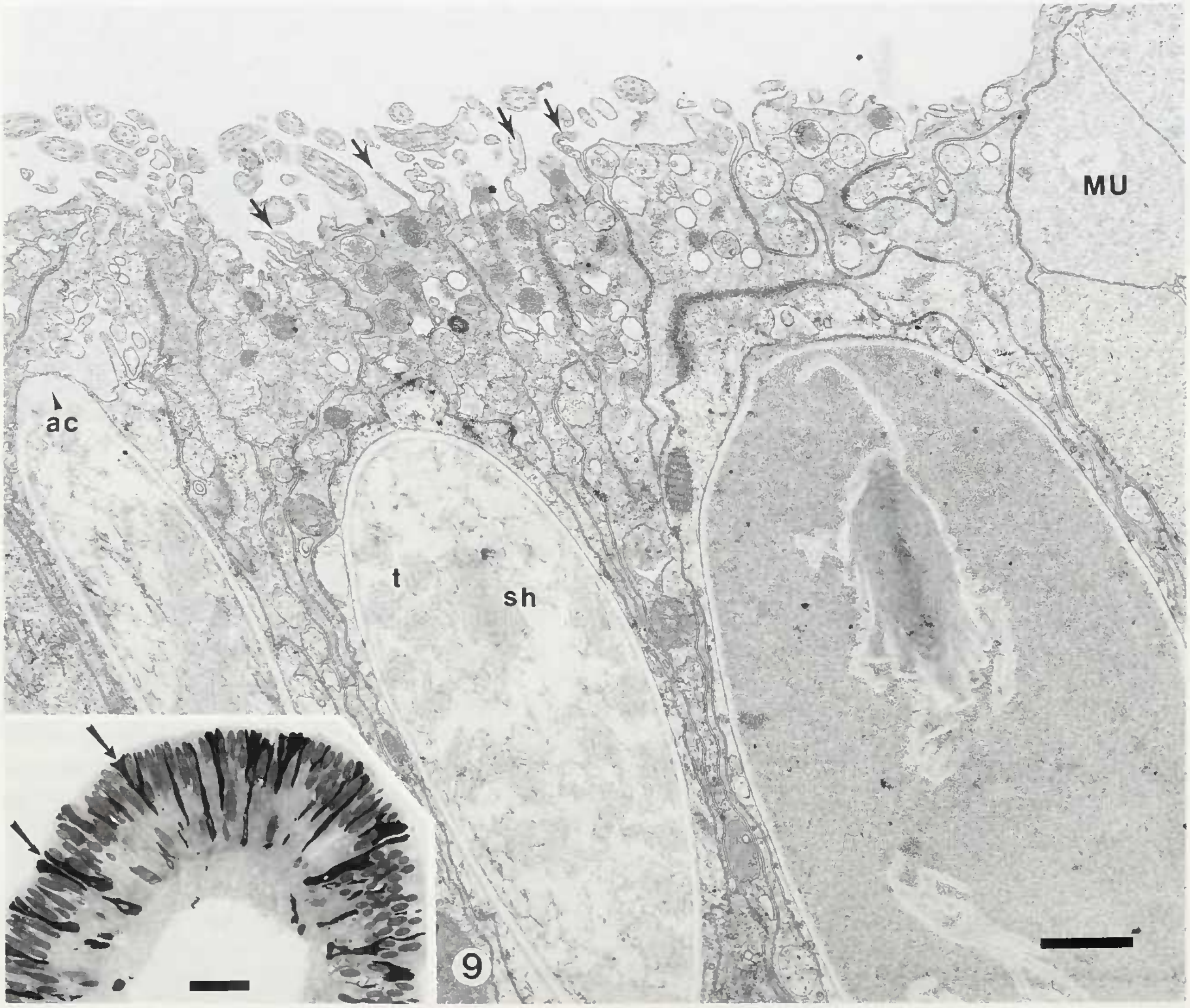


Figure 6. SEM preparation of sweeper tentacles. Note apparent absence of wart and microappendage structure. Surface cracks are artifacts of preparation. Scale bar = 50 μm. **Figure 7.** Higher magnification shows that surface ciliation occurs, but is greatly reduced. The center of a single wart (W) is indicated by

coelenterate material, or the possibility that algal colonization was secondary.

Sweeper tentacles in these black corals are extremely fragile, especially after fixation and dehydration. Except for artifactual cracks, their surfaces appear smooth, without the wart-like structure of normal tentacles (Fig. 6). At higher magnification, low mound-like warts with hirsute projections at their centers can be distinguished (Figs. 7, 8). The cells in the wart center are primarily cnidocytes, the surfaces of which form short microvilli and have a central cilium usually 3 μm or less in length (Fig. 9). The microvilli often form a collar-like arrangement at the tentacular surface (Fig. 8 inset), but ciliary cones, as such, apparently are absent (see Discussion).

Histologically, sweeper tentacles are completely different from their normal counterparts. The wart margins are still defined by mucus cells, but in sweeper tentacles these cells tend to be longer, narrower, and less numerous than those in normal tentacles (compare Figs. 3 inset and 9 inset). In addition, mucus cells stain more uniformly and produce a more intense pink metachromasia compared to their normal counterparts. More importantly, spirocysts are absent. All of the cnidae in the warts are nematocysts, and nearly all of them stain rapidly and deeply with Toluidine blue, giving the sweeper tentacle a comparatively uniform structure (Fig. 9 inset). Staining time must be held to less than 5 s at room temperature to avoid overstaining these cnidae. The capsules are 3.5 ± 0.5 μm wide and 22.5 ± 2.5 μm long. This is 40% wider and 25% longer than the dominant nematocyst found in normal tentacles. Warts generally contain 20–30 mature cnidae each, half or less of the 50–60 cnidae (mostly spirocysts) found in normal tentacles (Goldberg and Taylor, 1898a). Conversely, there is a greater number of warts per unit surface area in sweeper tentacles. In the boxed area of Figure 7, for example, 11 to 12 warts can be distinguished, whereas a comparable area from normal tentacle contains little more than two warts. Thus, sweeper tentacles contain approximately twice the number of cnidae per unit surface area.

Transmission electron microscopy shows that the structure of the shaft and capsule in the sweeper tentacle nematocysts are very similar to the type A MbMs described above from normal tentacle. However, differences in these cnidae become evident as they mature. The matrix becomes electron-lucent, tends to infiltrate poorly, and often shatters during sectioning. The shaft, matrix, and capsule wall usually lack adequate contrast (Fig. 9) even after overstaining. Occasionally, large nematocysts with a granular matrix can be found in or near final position (Fig. 9). These appear to be the same gastrodermal-type mastigophores that constitute 2.6% of the cnidae in the normal tentacle, but account for less than 1% of the sweeper tentacle nematocysts. These large cnidae also occur in the body wall epidermis of both normal and sweeper polyps. The latter appear to possess more of the gastrodermal cnidae than the sweeper tentacle, but proportionately no more than normal tentacle or body wall. Unlike normal polyps, however, the body wall is devoid of spirocysts. This condition indicates that the cnidae in the epidermis surrounding the sweeper tentacle are modified along with the tentacle itself.

Discussion

A few studies of sweeper or catch tentacle formation have included quantitative observations of the cnidae (Table I). In all but one of the specialized tentacles studied to date, spirocyst production is completely or almost completely suppressed in favor of nematocysts. In *Galaxea fascicularis* there is a 50% reduction in the spirocyst population of the sweeper tentacle acrosphere (Hidaka and Yamazato, 1984). Because spirocysts function in adhesion during food capture (Mariscal, 1974; Mariscal *et al.*, 1977), a change in favor of nematocysts is consistent with a change in tentacular function. However, a reduction in the development of surface microappendages in sweeper tentacle might not be predicted with the change from spirocysts to nematocysts. Mariscal *et al.* (1976) found microvilli, but no ciliary structures associated with spirocytes in several anthozoan species, and suggested that this may be an additional distinction between spirocytes and nematocytes. To the contrary, the normal tentacles of *A. fiordensis* are dominated by spiro-

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the arrow. Scale bar = 20 μm. **Figure 8.** Triple magnification of boxed area in Figure 7 shows reduced wart-like structure. Scale bar = 5 μm. Inset: surface detail showing part of a single wart. Clustered microvilli suggest ciliary cones, but a prominent central cilium is not apparent. Scale bar = 5 μm. **Figure 9.** TEM preparation showing typical appearance of sweeper tentacle epidermis. The only cnidae are microbasic b-mastigophores. The two at left are the type that constitute >99% of the nematocysts, accompanied by an occasional large, gastrodermal-type microbasic b-mastigophore at right. The dominant cnida has an apical cap (ac), a triply pleated tubule (t), and distinct shaft (sh) in a poorly infiltrated, electron-lucent matrix. The rim of the sweeper tentacle wart is formed by mucus cells (MU). The surface of each cnidocyte is drawn into a short collar of microvilli (arrows) that surround a single cilium. Scale bar = 1 μm. Inset: mucus cells in sweeper tentacles tend to be elongated (arrows), and stain intensely with Toluidine blue, as do the dominant nematocysts, giving the sweeper wart structure a totally different appearance in the light microscope. Scale bar = 20 μm.

TABLE I

Quantitative distribution of cnidae types in normal and modified tentacle

Taxon	Normal tentacle cnidae	Sweeper or catch tentacle cnidae
Scleractinia: *Galaxea fascicularis*[a]	70% Sp[1], 15% MpM[2] in acrosphere; HI[4] in tentacle and column with some MpM & Sp[4]	50% MbM[3], 35% Sp in acrosphere; MbM in tentacle with some HI and Sp
Scleractinia: *Montastrea cavernosa*[b]	83% Sp, 8% MbM in acrosphere	63% HI, 30% interm[5] in acrosphere
Actiniaria: *Metridium senile*[c]	82% Sp, 17% MbM	58% HI, 38% AI[6]
Actiniaria: *Haliplanella luciae*[d]	57% Sp, 26% MpM	99+ % HI
Antipatharia: *Antipathes fiordensis*[e]	89% Sp, 9% MbM	all MbM

[a] Hidaka and Yamazato, 1984.
[b] den Hartog, 1977.
[c] Purcell, 1977.
[d] Watson and Mariscal, 1983a.
[e] This paper
[1] Spirocyst = Sp.
[2] Microbasic p-mastigophore = MpM.
[3] Microbasic b-mastigophore = MbM.
[4] Holotrichous isorhiza = HI.
[5] Cnidae intermediate in form between HI and MbM.
[6] Atrichous isorhiza = AI.

cytes that not only possess well-developed microvilli, but are clearly ciliated (flagellated) as well (Goldberg and Taylor, 1989a). The distinct reduction in the hirsute appearance of its sweeper tentacles is therefore consistent with the absence of spirocytes. Further reduction in surface microappendage formation might result from a general decrease in epidermal microvilli. Because microvilli may serve as a reserve pool of membrane (Erickson and Trinkhaus, 1976), they might assist in the formation of elongated structures in coelenterates (Eppard *et al.*, 1989) including sweeper tentacles. Similarly, the absence of typical ciliary cones may be due to the shortness of the central cilium in the MbMs. Therefore, the absence of these putative receptor structures (*cf.* Mariscal, 1974) may be more apparent than real. Indeed, Hidaka and Miyazaki (1984) have suggested that ciliary cones may be typical of microbasic-b or p-mastigophores, as opposed to other nematocyst types. The presence of occasional ciliary cone structures in normal *A. fiordensis* tentacles may correspond to the lower relative abundance of these cnidae compared to sweeper tentacles.

The change in nematocyst type on conversion to sweeper tentacle appears to be inconsistent across taxa. Some species replace MpM (microbasic p-mastigophores) or HI (holotrichous isorhizas) in feeding tentacles with MbMs (microbasic b-mastigophores), whereas others replace MbM or MpMs with HIs, among others (Table I). In the case of *A. fiordensis*, the change in the nematocyst population involves differences in mean capsule size, appearance of the mature matrix, and staining characteristics. Other characters, especially the appearance of the shaft, suggest that the sweeper tentacle cnidae remain as MbMs. However, this designation is tentative due to the relatively small population of mature cnidae that remain undamaged after sectioning. Changes in the size, shape, and other details of nematocyst structure have been documented during the conversion to sweeper tentacles in other species (den Hartog, 1977; Hidaka *et al.*, 1987), as well as between metagenetic and ontogenetic stages of the same species (Fautin, 1988, and references therein). Some of these distinctions may be a function of taxonomy. Hidaka *et al.* (1987) discuss the problems of classification when attempting to distinguish populations of MbMs, basitrichs, and holotrichous nematocysts. They agree with Schmidt (1974), who concluded that intergrades exist between MbMs and basitrichs, and refer to both as b-rhabdoids. The inconsistencies listed in Table I may be resolved by lumping morphological characters of the shaft and tubule, but this may not address important functional changes that could be represented by increased capsule size (Watson and Mariscal, 1983a; Hidaka and Yamazato, 1984; Hidaka *et al.*, 1987; this paper) and changes in the physical properties of the nematocyst matrix (this paper) that accompany the conversion of normal to sweeper or catch tentacle.

Several authors have correlated the morphogenetic pattern of catch tentacle development with the formation of its cnidae (Purcell, 1977; Watson and Mariscal, 1983a; Hidaka and Yamazato, 1984; Hidaka *et al.*, 1987). In our study, sweeper tentacles irrespective of size, had already formed at the time of sampling. Thus, we have not had an opportunity to observe changes presumably occurring in the cnidom over time. Watson and Mariscal (1983b) showed that in *Haliplanella luciae*, catch tentacles not only exhibited a change in the type of cnidae, but displayed a maturational gradient in cnidae development as well. The catch tentacle base contained 96.3% cnidoblasts (cells with immature cnidae). Conversely, the catch tentacle tip contained mature cnidae almost exclusively. The opposite pattern was seen in feeding tentacles, where the gradient in maturity occurred cross-sectionally, *i.e.*, cnidoblasts were distributed along the base of the epidermis, while mature cnidae were restricted to the outer epidermis. These opposing patterns were not present in the sweeper tentacles of *A. fiordensis*. In both the normal tentacle (Goldberg and Taylor, 1989a) and the sweeper tentacle, nematocyst development took place in a cross-sectional gradient, not a longitudinal one. Whether this is a general distinction between sweeper and catch tentacles, or simply a distinction between two species, remains unknown.

Acknowledgments

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Parasitism and the Movements of Intertidal Gastropod Individuals

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Abstract. Movements of marked individuals of *Ilyanassa obsoleta* (n = 500) were charted in an intertidal environment for about one week. At the end of observations, 260 marked individuals, which had been sighted 1017 times collectively, were recollected and examined for trematode infections. Six trematode species were found in 19 infection combinations including uninfected, singly, doubly, and triply infected snails. We know that most snails found high on beaches and on sandbars carry *Gynaecotyla adunca* infections. It has been hypothesized that this host behavior modification is a parasite adaptation to enhance cercarial transmission to a semi-terrestrial next host. Observations reported here support this hypothesis and reveal some of the complexity in the behavior imposed on *I. obsoleta* by *G. adunca*. Individuals that were uninfected or infected with other parasites demonstrated no unique movement patterns, but individuals infected with *G. adunca* made repeated excursions into the upper shore habitat. These excursions were timed so that host-parasites were left emerged at high elevations primarily during nighttime low tides. Because many snails were multiply infected, data presented support the idea that gastropod populations have the potential to be used as systems for the study of the nature of ecological and evolutionary interactions among parasite species.

Introduction

Spatial and temporal patterns associated with intertidal animals have been studied by many authors. A recent review (Foster *et al.*, 1988) discusses the ecological factors thought to cause these patterns. However, pointing to a shortage of specific studies, these authors expressly (p. 13) did not include the effects of parasitism in their discussion, although they noted that the effects of parasites on their hosts can be substantial. Several studies have examined the movements of intertidal gastropods (*e.g.*, Underwood, 1977; Borowsky, 1979; Hazlett, 1984), but parasitism has not often been considered an important factor in these studies. Gastropods are an important component of marine and estuarine benthic systems. That parasites should not be ignored in these systems has been shown by previous studies with the mud snail *Ilyanassa obsoleta* and its trematode parasites (Sindermann, 1960; Stambaugh and McDermott, 1969; Curtis and Hurd, 1983; Curtis, 1985, 1987). The significance of parasitism has also been shown for at least one other abundant gastropod, *Littorina littorea* (*e.g.*, Sindermann and Farrin, 1962; Lambert and Farley, 1968; Williams and Ellis, 1975).

In a previous observation (Curtis, 1987) I noted a relationship between trematode parasitism and vertical zonation of the mud snail *Ilyanassa obsoleta* on the shore. Snails found high on beaches and on sandbars were usually parasitized by *Gynaecotyla adunca*. Snails unparasitized or parasitized with other species remained at lower levels. Data were interpreted to mean that the parasite alters the normal behavior of the host snail to enhance transmission of cercariae to semi-terrestrial (beach-dwelling) crustacean second hosts. Many parasites alter host behavior to enhance host-to-host transmission by means of predation [see Dobson (1987) for references], but this is apparently the first observation suggesting altered behavior with the adaptive advantage of enhancing cercarial transmission.

Thus far, inferences concerning the behavior-altering

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capacity of *Gynaecotyla adunca* have been drawn from correlative population-level data with no attempt to determine the behavior of individual snails. Observations of individual host behavior are necessary because one cannot determine the exact composition of the altered behavior by comparing parasitism in populations at different vertical levels on the shore (Curtis, 1987). Many questions are left unanswered by this approach. Does the same host repeatedly move onto the same area of beach or sandbar? If repeated visits occur, is there a schedule? Do hosts always move to a similar vertical position? Does this mean that *G. adunca*-infected snails move around in the habitat significantly more than other snails? In general, just how simple or complex is the altered behavior imposed on *Ilyanassa obsoleta* by this parasite?

Data presented in this paper help address these questions. Positional histories of individually marked snails with various infections (including uninfected) were charted in a natural sandflat environment for approximately one week. Data indicate that *Gynaecotyla adunca*-infected snails, despite the presence or absence of co-occurring trematodes, have a complex, patterned behavior that is much different from the behavior of snails when unparasitized or parasitized by other trematodes. In a broader sense, the results illustrate the depth to which parasites become insinuated into ecological systems and their contribution to the complexity of those systems. Additionally, the high frequencies of double and triple infections revealed by this (and other) work support the idea that host gastropod populations could be more useful as systems for the study of parasite species interactions than is currently appreciated.

Materials and Methods

The experiment took place during July 1985 on the Cape Henlopen sandflat in Delaware Bay (described in Curtis and Hurd, 1983). The same sandbar area that had been used in 1984 (Curtis, 1987) was used again. The configuration and position of the sandbar had changed somewhat over the winter, so on 2 July sandbar topography was again mapped (Fig. 1) using the methods described in Curtis (1987). A 3 × 5 m plot was marked out at the peak of the sandbar and a pipe was driven deep into the center of the plot as a vertical (zero elevation) and horizontal reference point for measurements made during the experiment (see Fig. 1).

Cape Henlopen has two low and two high tides per day that are roughly equal in range (*ca.* 1–1.5 m). The sandbar peak was emerged for approximately 3 h on either side of low tide and submerged for approximately 3 h on either side of high tide.

In 1984 there was a clear correlation between proportion of snails parasitized by *Gynaecotyla adunca* and elevation. To determine whether this was also true in 1985, snails were collected from the sandbar peak area (n = 185) and, for comparison, from 11 sites on the sandflat peripheral to the sandbar (n = 125). Sandflat snails were collected from areas at least 28 cm down from the sandbar plot (Fig. 1). These were examined for size, sex, and parasitism as described in Curtis (1985).

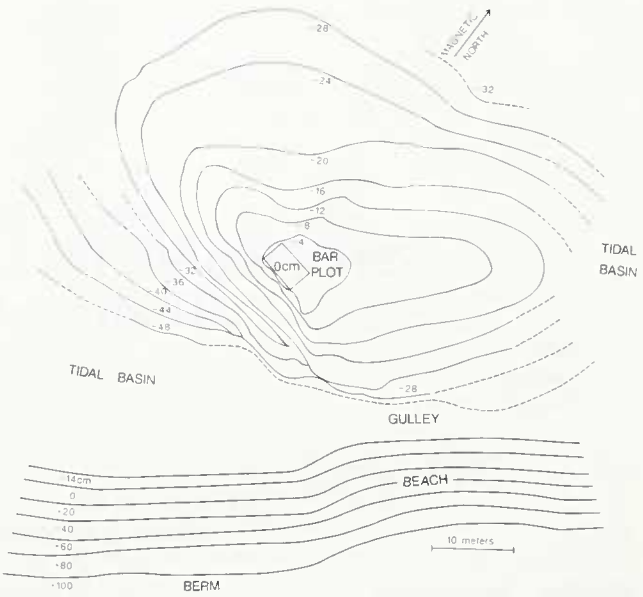

Figure 1. A sketch map of the Cape Henlopen sandbar used for this study. Zones of elevation and position of the 3 × 5 meter plot are shown.

Generally, the design of the experiment involved collecting two sets of snails: sandbar/beach snails (which were likely to be infected with *Gynaecotyla adunca*); and sandflat snails (which were unlikely to be so infected). Snails in both sets were marked individually and released on the sandbar. The sandbar, adjacent sandflat, and beach were searched for marked individuals during each subsequent low tide for about one week. As marked individuals were located, their positions relative to plot center were recorded using polar coordinates.

More specifically, snails were marked with numbered insect tags glued onto a dried and roughened shell area with clear fingernail polish. Sandflat snails (n = 250) were collected on 15 July during the 1309 h low tide. (All times are Eastern daylight savings time.) They were measured for size, marked, and retained in the laboratory until release. Sandbar/beach snails (n = 250) were collected during the 0216 h low tide on 16 July. They were measured, marked, and retained in the laboratory. On the next low tide (16 July, 1354 h) the 500 marked snails were released on the sandbar in 20 groups of 25 (13 sandbar/beach snails plus 12 sandflat snails, or the reverse) placed at equal intervals along a circumference

Table I

Nearby physical conditions and numbers of snails at the sandbar peak (3 × 5 m plot) during the 16–24 July 1985 Cape Henlopen experiment with individually marked Ilyanassa obsoleta

Low tide #	Time EDST	Lighting	Ta	Tw	Sal	Snails in plot	% marked
0	1354	L	25	24	29	(marked snails released)	
1	0257	D	24	23	28	42	12
2	1437	L	27	28	?	4	25
3	0339	D	21	21	?	63	40
4	1522	L	29	32	28	15	27
5	0418	D	18	19	18	102	37
6	1608	L	24	28	29	3	33
7	0500	D	23	21	27	70	36
8	1655	L	28	30	30	7	0
9	0543	D	25	23	24	27	48
10	1745	L	26	26	26	3	67
11	0627	D	24	24	26	45	40
12	1840	L	26	26	29	15	47
13	0713	D	20	21	?	39	36
14	1938	D	23	23	30	14	27
15	0803	L	—	—	—	—	—
16	2040	D	—	—	—	—	—

Abbreviations: Ta and Tw = air and water temperature (°C); sal = salinity (g/kg); and lighting conditions (L = light, D = dark). A tide is labeled "dark" if it occurred between sunset minus one hour (1900 h) and sunrise plus two hours (0800 h).

at a 10-m radius from the plot center (Fig. 1). On this and all subsequent low tides during the experiment, all snails inside the plot were evicted (scattered just outside the plot). Thus on any given low tide, counts of plot snails reflected those that had moved in during the previous high water and remained.

The procedure for locating marked snails involved searching the sandbar and its periphery. Whenever a marked snail was sighted, mark number, time, meters from plot center, and number of degrees clockwise from magnetic north were noted. The sandbar and adjacent sandflat were searched in ever wider circles until water became too deep (about 15 cm) to observe snails. Depending on tidal range, the sandbar and adjacent areas could be effectively searched for up to three hours on one low tide. If the release tide is tide zero, searches were performed and snail positions noted through tide 16 (24 July, 2040 h). Most marked snails were recollected on tides 14–16. Many marked snails were recollected after tide 16 (through 28 August). Sightings of these snails during the experimental week were used, but positions recorded at recollection were not.

By transferring position of a snail at sighting to the contour map of the sandbar (Fig. 1) elevation of the snail at that sighting and net distance moved since the previous sighting were determined. Snail elevations were assigned to discrete elevation zones (0, 2, 4, 6 · · · 50 cm below plot center). A mean elevation at sighting was calculated for each recovered marked snail. Net distance moved per tide by a snail between sightings was determined by converting polar coordinates from field measurements to rectangular coordinates, calculating distance between the two points and dividing by the number of elapsed tides. To reduce any effects of the marking and release procedure, movements between points of release and first sightings were excluded. A mean net movement per tide (rounded to the nearest meter) was calculated for each recovered marked snail that was observed at least twice. Recovered snails were examined in the laboratory by dissection so individual snail histories during the experiment could be compared based on parasitism and other characteristics. Comparisons among snails with different infections in terms of elevation on the sandbar and net distance moved were made using nonparametric statistical methods (Sokal and Rohlf, 1981).

Results

Table I shows some of the physical conditions prevailing on low tides during the experiment as well as numbers of snails entering the 3 × 5 m plot at the sandbar peak during the previous high water. Temperatures and salinities were in the usual ranges for Cape Henlopen. No information is given for tides 15 and 16 because from tide 14 on snails were being collected as sighted. In Table I, tides are classified as occurring under light or dark conditions, and it is apparent that more snails were present in the plot on dark low tides (mean = 50.25, S.D. = 27.53) than on light low tides (mean = 7.83, S.D.

Table II

Statistics for shell size and sex ratio, and number of snails in each trematode infection category, for groups of Ilyanassa obsoleta *collected and examined in connection with the snail movement experiment*

Parameter	Unmarked snail samples: Sandbar peak	Unmarked snail samples: Sandflat	Recollected (marked) snails
Shell height (mm)			
mean	21.1	20.3	21.4
S.D.	1.7	2.3	1.5
% female:% male	47:53	50:50	44:56
Infection*			
NI	39	69	32
Hq	0	10	8
Ls	0	4	5
Zr	2	35	19
Av	0	2	5
Ga	78	0	106
Dn	0	1	1
HZ	1	1	3
HG	0	0	3
LZ	1	1	4
LG	22	1	31
ZA	1	0	0
ZG	25	0	30
AG	5	0	6
GD	1	0	0
GSt	0	0	1
LZG	5	1	3
LAG	2	0	3
ZAG	1	0	0
TOTALS	185	125	260
% Ga-infected	75.1	1.6	70.4

* Abbreviations: NI = not infected; Hq = *Himasthla quissetensis;* Ls = *Lepocreadium setiferoides;* Zr = *Zoogonus rubellus;* Av = *Austrobilharzia variglandis;* Ga = *Gynaecotyla adunca;* Dn = *Diplostomum nassa;* St = *Stephanostomum tenue;* multiple infections are abbreviated with the generic initials of the species involved (*e.g.*, ZAG = ZrAvGa triple infection).

= 5.74). Table I further shows that on most tides many snails in the plot had marks, which suggests that they made up a substantial proportion of total snails using the sandbar. Table II shows that the groups of snails examined in this study did not differ significantly in terms of age (shell height) or proportions of males and females. However, it is clear that snails visiting the sandbar peak (plot) were largely *Gynaecotyla adunca*-infected, whereas snails from the adjacent sandflat were not (Table II, unmarked snails). Table II also shows the variety of multiple infections present and, in particular, the large proportion of *G. adunca* infections that were combined with other trematode infections: of the 324 observed *G. adunca*-infected snails in Table II, nearly half (43.2%) were multiply infected.

Of the 500 snails marked and released, 260 were ultimately recovered. Frequencies of trematode infections among recollected marked snails, which were sighted 1017 times collectively over the 16 tides, are shown in Table II. Based on initial collection site, there were two groups of marked snails: a sandbar/beach group (n = 250, mean shell height = 21.5 mm, S.D. = 1.5); and a sandflat group (n = 250, mean shell height = 20.8 mm, S.D. = 1.5). The likelihood of sighting and recovering a snail was clearly different for individuals in the two groups: 74.6% of the sandbar/beach snails were recovered (93.6% were sighted at least once after release); only 31.2% of the sandflat snails were recovered (68.4% were sighted after release). Almost all (99.5%) recovered snails from the sandbar/beach group (n = 182) had *Gynaecotyla adunca* infections, while very few (2.6%) recovered snails from the sandflat group (n = 78) had this parasite. Marked snails apparently stayed near the sandbar during the experiment because regular searches of the adjacent beach (Fig. 1) and a neighboring sandbar 30 m SW revealed no marked snails. The less frequent observation and greater loss of marked sandflat group snails can be explained by their being dispersed in a larger area (see below).

Three marked snails were sighted in the area of the 1985 sandbar during July and August 1986. By this time, the experimental sandbar no longer existed. Snail A was uninfected when examined and had a shell height of 20.0 mm (19.9 mm when marked). It was observed twice in 1985 around the periphery of the sandbar. Snails B and C were both double infected with *Zoogonus rubellus* and *Gynaecotyla adunca* and had shell heights of 24.9 and 23.8 mm (24.7 and 23.7 mm, respectively, when marked). Both snails had been sighted several times (total = 8) on the peak of the sandbar in 1985. Such data are subject to multiple interpretations, but indications are: (1) that growth for these large snails was slight during the previous year; and (2) based on behavior, snail A was probably not infected in 1985 and remained so, while snails B and C were both infected with (at least) *G. adunca* in 1985 and had carried their infections through the winter.

Sightings of marked snails during the experiment provided information on elevation at sighting and movement between sightings for individuals with various single and multiple trematode infections. Because *Gynaecotyla adunca*-infected snails aggregated near the sandbar peak, probability of observing these snails was enhanced. Snails that did not behave this way were dispersed in a larger area (the outer edge and periphery of the sandbar), and the probability of observing them was reduced. Activities of *G. adunca*-infected snails would be over-represented in the data if all the individual sightings (range = 1–9/snail) were used as datum points. To correct for

Table III

Relationship between trematode parasitism and elevation at sighting (cm down from bar peak) of marked Ilyanassa obsoleta *individuals on a Cape Henlopen sandbar**

	Parasitism								
Parameter	NI	Hq	Ls	Zr	Av	Ga	LG	ZG	AG
# marked snails	32	8	5	19	5	106	31	30	6
Total # sightings	78	17	18	53	14	460	151	115	34
Median of mean elevs. (cm)	38	34.7	30	38.7	42	5.5	4.7	4.5	4.5
Range (cm)	3–50	33–48	22–47	25–50	34–51	0–32	0–19	0–30	1–8
Avg. rank	201	205	195	209	219	93	75	88	79

* There was a significant effect of parasitism on the mean elevations of individual snails (Kruskal-Wallis test, H = 139.456, d.o.f. = 8, $P < 0.001$). Higher average ranks denote lower elevations. Abbreviations as in Table II.

this, I used mean elevation at sighting and mean net movement per tide between sightings as datum points for each snail. This procedure emphasizes infrequently sighted snails (*i.e.*, not *G. adunca*-infected). Neither tendency is desirable, but using the mean value for each snail leads to more conservative comparisons because statistical tests are not swamped with the behavior of frequently observed *G. adunca*-infected snails.

Categories of infection with fewer than five snails were eliminated from Tables III and IV. Table III shows that the elevation of *Ilyanassa obsoleta* at sighting was influenced by parasitism. Individuals infected with *Gynaecotyla adunca*, either singly or in multiple, were sighted at higher elevations on the sandbar (lower average ranks in the table) than uninfected or otherwise infected individuals. Table IV shows that net movement per tide of individual snails was also influenced by parasitism. It should be recognized that these measurements record minimum movement. Snails could not have moved less than the distances measured, but undoubtedly moved more. Average ranks and medians (Table IV) show that there were two groups of snails with respect to net movement per tide; those infected with *G. adunca* and those not. A statistical comparison of just these two groups confirms that *G. adunca*-infected snails moved around more than other snails (Mann-Whitney U Test, U = 5657.5, $P < 0.001$).

A long interval between pairs of sightings could affect the magnitude of calculated net movement per tide. With snails remaining in the same general area, as many did in this study, this would probably lead to an underestimation of net movement. If time between sightings was routinely greater for snails lacking *Gynaecotyla adunca* (which were seen less often) compared to those infected, this could introduce a bias and compromise the result (Table IV) that snails with *G. adunca* moved around more than other snails. The range of elapsed tides between sighting pairs was the same for both groups (1–13). The mean number of tides between pairs of sightings for all recollected *G. adunca*-infected snails (Table II) was 2.5, with 91% of sighting pairs (n = 625) coming four or fewer tides apart; the corresponding mean for recollected

Table IV

Relationship between trematode parasitism and mean net movement between pairs of low tides for individually marked Ilyanassa obsoleta *on a Cape Henlopen sandbar**

	Parasitism							
Parameter	NI	Hq	Ls	Zr	Ga	LG	ZG	AG
# marked snails	22	6	5	17	104	30	28	6
Total # net moves obs.	48	9	13	35	351	120	84	27
Median of mean net moves (m)	1.6	1.5	1.7	1.8	2.5	2.6	2.9	2.5
Range (m)	0–15	0–4	1–3	0–7	0–11	0–8	1–8	1–3
Avg. rank	75	83	67	90	114	121	130	113

* There was a significant effect of parasitism on net movement per tide of individual snails (Kruskal-Wallis test, H = 16.987, d.o.f. = 7, $P < 0.02$). Higher average ranks denote more movement. Abbreviations as in Table II.

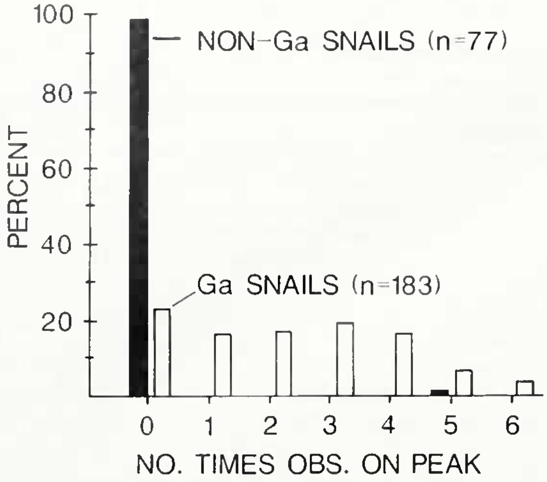

Figure 2. Frequencies of visitation to the sandbar peak (upper vertical 4 cm in Fig. 1) by *Ilyanassa obsoleta* infected with *Gynaecotyla adunca* (Ga snails) and those not infected (non-Ga snails).

snails without *G. adunca* was 3.4, with 75% of sighting pairs (n = 131) coming within four tides. Therefore, the usual number of tides between sightings was similar (1–4) for both groups of snails. Even if longer periods between sightings tend to reduce calculated net movements per tide, relatively few measurements would have been involved. Moreover, both groups of data would have been affected similarly. A biased underestimation, on this account, is unlikely.

Frequency of visitation to the sandbar peak by the 260 recovered snails (Table II) is shown in Figure 2. Snails not infected with *Gynaecotyla adunca* almost never showed up in the upper 4 cm area of the sandbar. Only a single snail not infected with *G. adunca* (recorded as uninfected) made multiple visits to the peak. Among *G. adunca*-infected snails, however, many individuals visited the peak several times (up to six) during the 16-tide experiment. Visitations could have been more frequent and regular than indicated (Fig. 2) if buried marked snails escaped notice on some tides. In any case, it is clear that many host snails made repeated excursions onto the same sandbar peak.

The behavior of *Gynaecotyla adunca*-infected snails has a diurnal component because they are most frequent on beaches and sandbars during night low tides (Curtis, 1987; Table I). The diurnal pattern of visitation to the sandbar peak by host individuals, which results in this observation, is revealed by the following data. The peak is defined here as the area within the 4-cm contour (Fig. 1). Among *G. adunca*-infected snails, 112 were observed on the peak two or more times. Most sightings for these (90%, n = 377) were on night low tides. None of these snails was sighted exclusively on daytime low tides; 72% were sighted exclusively on nighttime low tides and 28% showed up on both day and night low tides. Among the 28% seen on both dark and light tides, 71% of sightings were on dark tides. Therefore, host-parasites were typically emerged during night low tides with daytime emergence being relatively infrequent.

Among recovered *Gynaecotyla adunca*-infected snails, 77 were sighted on both light and dark low tides. Were they sighted at similar elevations on both types of tides? In a Wilcoxon Signed-Ranks Test for two groups (paired observations), these snails had a significantly higher elevation when sighted on the sandbar at night (mean = 7.0 cm down from peak, range = 0–34) than when sighted during the day (mean = 9.5 cm, range = 0–50) (Wilcoxon T = 869.5, $P < 0.002$). Therefore, not only did infected snails tend to make night visits to the sandbar, but on those night visits they positioned themselves higher than on day visits.

Discussion

My results show that *Ilyanassa obsoleta* infected with *Gynaecotyla adunca* exhibits a complex behavior unlike that of snails lacking this parasite. These snails make repeated migrations (Fig. 2) to the higher reaches (Table III; Curtis, 1987) of sandbars and beaches, and these excursions entail a generally greater amount of movement per tide than that demonstrated by other snails (Table IV). These migrations leave hosts emerged primarily (but not exclusively) during low tides that occur at night (Table I). When a host snail is sighted on both night and day low tides, it is usually found at higher elevations at night.

All this suggests that there is adaptive value to the parasite in repeatedly inducing its host to be located high on the shore during nighttime low tides. *Ilyanassa obsoleta* is the main first intermediate host for *Gynaecotyla adunca*. Definitive hosts include any of a variety of shore birds and certain fish (Hunter, 1952) and mammal species (Harkema and Miller, 1962). I proposed previously (Curtis, 1987) that the adaptive value lies in an enhanced probability of cercarial transmission to semi-terrestrial, crustacean second intermediate hosts [the amphipod beach-hoppers, *Talorchestia longicornis* (Rankin, 1940) and *T. megalopthalmia* (Hunter and Vernberg, 1957) or the fiddler crab *Uca pugilator* (Hunter, 1952)]. The present results support, and allow refinement of this general hypothesis.

Fiddler crabs do not inhabit Cape Henlopen, but beach-hoppers do. It should be noted, however, that these second hosts are beach (not sandbar) dwellers. Because the basic behavior of infected snails is the same on sandbars and beaches (Curtis, 1987), the two habitats are apparently indistinguishable by infected individuals. It is worth noting that, in adaptive terms, migrating up and down Cape Henlopen sandbars appears to be a waste of parasite time and energy because the next host is not found there. Presumably, however, over the geographical range of the parasite, a next host is present often enough that parasite fitness is enhanced by inducing host vertical migrations regardless of whether a second host is present or absent on a particular shore.

What cues could be used to control the migration? Based on field observations (Curtis, 1987), I concluded that the day-night difference in numbers of host-parasites on the sandbar is a matter of differential immigration during submergence, not differential emigration during emergence. Thus, when a sandbar or beach is examined at low tide, snails found there had moved into position hours before during the previous high tide. This means that the host-parasite tends not to move up the shore during a high tide to be followed by a daytime low tide, but tends to do so on a high tide that will be followed by a nighttime low tide (Curtis, 1987; present results). Therefore, a remarkable feature of the host-parasite is its ability to track and respond to high tides associated with appropriate future low tides.

From an adaptive standpoint, why avoid stranding the host on daytime low tides? Curtis (1987) noted that the diurnal difference in migratory behavior could be adaptive because (1) it matches diurnal behavior patterns of the next host or (2) it lessens the risk of desiccation to the host or the parasite. Beach-hoppers (Talitridae) tend to be active at night and burrow during the day (Kaestner, 1970; pers. obs.), making (1) above very probable. In this study, individuals infected with *Gynaecotyla adunca* were observed on daytime tides and later again on nighttime tides, which shows that daytime exposure is not lethal to the infection. Anyway, parasite stages within the host are probably not the ones at risk. More likely, cercariae cannot survive the rigors of exposure outside the host during daytime low tides. Tentatively then, host excursions onto a daytime beach would reduce the parasite's fitness for two reasons: the next host would not be around to infect; and released cercariae would not survive until hosts become active on a subsequent nighttime tide.

In studies where the effect of trematode parasitism on intertidal snail movement has been considered (Sindermann, 1960; Lambert and Farley, 1968; Stambaugh and McDermott, 1969; Williams and Ellis, 1975), the general conclusion has been that parasitism inhibits movement of the host. Perhaps parasitism does decrease gastropod movement in some circumstances, but in this study (Table IV) it only increased it. Snails infected with *Gynaecotyla adunca*, singly or in combination with other species, could be distinguished from the rest because, consistent with their migratory behavior, they exhibited greater mean net movement per tide. However, uninfected snails could not be distinguished from infected ones (except *G. adunca*-infected) based on movement. This result suggests that, during summer and in terms of locomotion in the field, *Ilyanassa obsoleta* is not significantly impaired by trematode parasites.

Gynaecotyla adunca is found in many multiple infections (Curtis, 1985, 1987, Table II) and has an overriding influence on the host regardless of the presence or absence of other trematodes (Fig. 2, Tables III, IV). This suggests that interactions with other trematode species are likely. Most accounts of ecological and evolutionary relationships among co-occurring helminths emphasize the interactions of adult parasites in vertebrates (*e.g.*, Price, 1980; Holmes, 1983, 1986; Holmes and Price, 1986). Holmes and Price (1986) and Holmes (1986) delineate three hierarchical levels of parasite assemblage organization: infra-, component, and compound communities. Data from this study (Table II) are, in themselves, probably too few for meaningful analysis, but in kind they stand to reveal organization at infra- and component levels.

The development of more host-parasite systems amenable to study is essential to progress in the field of parasite community (guild) ecology (Price, 1986). Larval trematodes in marine gastropods have not been much studied in this context because multiple infections are, or are considered to be, too infrequent (*e.g.*, see Vernberg *et al.*, 1969). Gastropod-trematode systems have been analyzed for parasite species interactions (see Rohde, 1981 and references therein) and co-occurrence interactions have been noted, but such systems have probably been underutilized. Field studies (Cort *et al.*, 1937; Rohde, 1981; Curtis, 1985, 1987) show that multiple infections can be commonplace. In this study, 570 snails were examined (Table II): 75.4% were infected (19 different combinations); 26.7% were multiply infected. *Ilyanassa obsoleta* individuals and populations should be added to the list of systems in which interactions among parasites can be studied.

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Geographic Variation in Naupliar Growth and Survival in a Harpacticoid Copepod

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Abstract. Newly hatched nauplii of *Scottolana canadensis* (Willey) collected from two locales in Maine were larger than Maryland nauplii when females were reared under identical conditions (20°C and high food concentration, 2.5×10^5 algal cells ml^{-1}). Under high food concentration, Maryland nauplii had faster growth rates ($\log_{10}$ μm h^{-1}) than Maine nauplii, but survivorship was similar. Growth rates were lower under low food concentration (0.5×10^5 cells ml^{-1}), and were the same for all locales, whereas survivorship of the Maine nauplii through NV was higher than the Maryland nauplii. We hypothesize that size-related differences in naupliar feeding efficiency may explain the variation in survival under low food stress.

Introduction

In many marine organisms with mechanisms for dispersal and hence the potential for gene flow, genetic differentiation of populations has occurred (*e.g.*, as found in copepods; Bucklin and Marcus, 1985; Burton, 1986; reviewed in Hedgecock, 1986). The potential for genetic differentiation of estuarine populations may be greater than that for coastal populations because of physical barriers to dispersal (*e.g.*, salinity and temperature; Koehn *et al.*, 1980; McAlice, 1981) and because selective forces arising from human activities may be more intense (*e.g.*, PCB pollution; Cosper *et al.*, 1984, 1988; see review by Levinton, 1980).

Scottolana canadensis (Willey; but see Por, 1984) is a widespread, brackish-water harpacticoid copepod (Willey, 1923; Haertel *et al.*, 1969; Coull, 1972). When common-rearing techniques were used in the laboratory, females from Maine (43°N) produced larger eggs that took longer to develop at all test temperatures than those from Maryland (38°N) and Florida (27°N; Lonsdale and Levinton, 1985a). These results were contrary to the expectation that northern-derived populations would demonstrate compensation for low temperature. Furthermore, low food stress (2.5×10^4 algal cells ml^{-1}) produced locale differences in newborn survival, with Maine nauplii surviving in the highest proportion. Although variation in egg size does not necessarily reflect differences in egg organic content (McEdward and Carson, 1987), the correspondance between large egg size and increased survival of newborns suggested that the Maine females produced eggs with more yolk than did females from other locales, resulting in differences in maternal reserves for newly hatched nauplii (Lonsdale and Levinton, 1985a).

The Saco River, from which the Maine *S. canadensis* individuals were collected, is characterized by extremely high rates of freshwater flow (B. McAlice, pers. comm.), and Saco Bay receives "sediment input grossly out of proportion to (its) size" (Kelly *et al.*, 1986; cited in Jacobson *et al.*, 1987). The planktonic nauplii of *S. canadensis* would probably reach their physiological limits once carried into the Gulf of Maine due to rapidly declining temperatures (McAlice, 1981) and possibly to increased salinities. Laboratory studies have shown that the survivorship of *S. canadensis* is adversely affected by higher salinities (responses to 10, 15, and 20‰ were studied; Lonsdale, 1981), but the differences were largely found in the epibenthic copepodites rather than the planktonic nauplii. Thus, the efficacy of salinity as a barrier to dispersal in the species is moot.

In this paper, we present the results of a test of the "nauplius development time restriction" hypothesis; *i.e.*, that "yolkier eggs may enhance the rate of nauplius development to Copepodite I, at which stage they migrate

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Table I

Location and physical characteristics of collection sites for Scottolana canadensis

Collection site	Date	Temperature (°C)	Salinity (‰)
Sheepscott River (SHP), Wiscasset, Maine	May 1988	17	12
Saco River (SR), Biddeford, Maine	May 1988	13	10
Patuxent River (MD), Lusby, Maryland	May 1988	18	10

to the bottom, thereby increasing the probability of *S. canadensis* remaining within the (Saco River) estuary" (p. 428; Lonsdale and Levinton, 1985a). Thus, the egg and naupliar traits of the Maine copepods may not have reflected a latitudinal trend, but rather a localized evolution to a unique hydrodynamic condition. To test the hypothesis, we compared egg development time, egg size, and naupliar growth and survival rates of *S. canadensis* collected from the Saco River (SR) and Sheepscott River (SHP) estuaries in Maine, and the Patuxent River estuary in Maryland (MD).

Materials and Methods

Field collections

Planktonic nauplii of *Scottolana canadensis* were obtained with a 63 μm-mesh net from three sites on the east coast of North America; pertinent collection information is listed in Table I. Separate collections consisted of 250 or more nauplii. Specimens of *Scottolana* collected from Maine and Maryland are interfertile (Lonsdale *et al.*, 1988).

Culture methods

In the laboratory, wild-caught copepods were cultured at 20°C in 1000-ml Erlenmeyer flasks containing 15‰ seawater. Seawater was prepared with water from Stony Brook Harbor, New York (~27 ‰), adjusted to 15‰ with distilled water, filtered through 20-μm mesh, and autoclaved. Algae also were cultured at 20°C and 15‰ with a 14:10 hour light-dark cycle in f/2 enrichment medium (Guillard, 1975), and maintained in an approximately log phase of growth by harvesting and adding medium three times weekly. Generally, algal cultures used both in copepod culturing and experiments ranged in age from 5 to 14 days. Cell densities were assessed with a hemocytometer. A mixture of two algal species—*Isochrysis galbana* (ISO; ~4–6 μm diameter and ~14.1 pg C cell^{-1} according to the Strathmann equations; Strathmann, 1967) and *Thalassiosira pseudonana* (3H; ~4 μm and ~9.0 pg C cell^{-1})—was added to each copepod culture three times weekly to produce a minimum density of 2.5×10^5 cells ml^{-1}. Copepod batch cultures from each locale (SHP, SR, MD) were maintained for at least two months prior to experimentation.

Experimental procedures

Egg development times. Seventy-five nauplii (all stages) from each locale were removed from batch culture and reared in 1000-ml beakers using the above methods. These and all other experiments were conducted at 20°C. At this temperature, copepods from Maine and Maryland exhibited little difference, either in mean growth rate from nauplius I to adult, or in adult female energy budgets (Lonsdale and Levinton, 1985b, 1986, 1989). Several additional cultures of SHP nauplii were set up because the first had produced many non-reproducing females, possibly due to an infectious agent (see Lonsdale and Levinton, 1986).

Following maturation, up to 30 gravid females were selected and individually placed in 50-ml covered Stendor dishes. Each dish contained about 20 ml of sterilized seawater to which algae (a 1:1 mixture by cell number of *I. galbana* and *T. pseudonana*) was added to bring the food concentration to 2.5×10^5 cells ml^{-1}. Females were fed three times weekly, and the seawater was changed once a week. To further minimize culture effects on egg traits, development times were determined only after the female produced a second or third clutch. The third clutch was studied in the case of females already carrying egg sacs. Usually, these females were producing additional eggs visible in the oviducts.

To estimate egg development times, observations were made at 4-h intervals. Total time (h) was calculated from the extrusion of eggs in sacs, to naupliar hatching.

Egg volume. To determine whether females collected from the Saco River estuary produced larger eggs than those from the Sheepscott River and Patuxent River estuaries, females with egg clutches were preserved according to the procedures outlined by Gallager and Mann (1981). To determine whether changes in egg volume are associated with embryogenesis, some females were preserved within 4 h of clutch formation (t = 0 h) and others after 24, 48, 72, or 96 h (for each locale and time condition, n = 3–4 females except n = 2 for SHP at 96 h due to the low number of fecund females). For each clutch, dimension measurements were made on six eggs. Egg volume was calculated (after Allan, 1984) according to the formula: volume (μm^3) = $\frac{4}{3} \pi r_1 r_2^2$, where r_1 and r_2 are the radii of the long and short dimensions, respectively. Egg dimensions and lengths of females (μm) were measured with an Optical Pattern Recognition System

Table II

Mean (95% confidence interval) CI length (μm) and total development time (h) to CI for Scottolana canadensis *collected from three locales (SR, SHP, and MD) and reared at 20°C and two food concentrations (2.5 and 0.5 × 10^5 cells ml^{-1})*

Food	Locale	Length	Development time
2.5	SR	341 (330–352)	112 (106–118)
	SHP	329 (318–341)	112 (105–120)
	MD	313 (302–324)	103 (96–111)
0.5	SR	308 (301–316)	164 (135–201)
	SHP	297 (268–329)	163 (136–195)
	MD	293 (278–309)	141 (110–180)

SR = Saco River; SHP = Sheepscott River; MD = Patuxent River.

(Biosonics, Inc.) at 400 × enlargement under a Zeiss compound microscope. Female dry mass was estimated by the equation: $Y = 8.415\ X^{1.957}$, where Y = μg dry mass, and X = length in mm (Lonsdale and Levinton, 1985b).

Naupliar growth and survival. Newborn nauplii, obtained from the egg development time studies, were placed individually in wells of a multi-depression dish that was contained within an airtight opaque plastic box. Distilled water in the bottom of the box reduced evaporation from the wells. Four sibs from five (SHP) or six families (SR, MD) were followed at each food concentration (0.5 and 2.5 × 10^5 cells ml^{-1}). The carbon concentration of the algal suspensions (~577 and 2887 μg C l^{-1}, respectively) were within the range found in many estuaries (*e.g.*, 240–3910 μg C l^{-1} for East Lagoon, Texas (Ambler, 1986) and 500–3388 μg C l^{-1} for Narragansett Bay (Durbin *et al.*, 1983). Six additional sibs were preserved in 5% buffered formalin for measurements of body dimensions (length and width) that were determined at 100× enlargement with a Wild inverted microscope. (Nauplii from one SHP family were inadvertently not sampled for measurement.) The algal suspensions in the wells were completely replaced daily at 1200 h, and a 50% replacement occurred at 2400 h. The algal suspensions were prepared fresh each day from sterilized, filtered seawater (15‰) and algae. The copepods were observed about every 4 h, and the time to each stage (NII to CI) was noted. Molt lengths were measured during the 100% medium replacement.

Equality of variances in the data sets was tested using the F_{max}-test and, if necessary, the data were $\log_{10}$ transformed prior to analysis of variance (Sokal and Rohlf, 1981). Most statistical tests were conducted using the packages of Sokal and Rohlf (1981) or SAS.

Results

Egg development time

A significant difference in egg development time was found among locales (One-way ANOVA; df = 2,27, F = 17.03, $P < 0.0001$). Mean egg development time (95% confidence interval) of MD females was significantly less than that of SR and SHP females [74.1 (72.3–75.9) h *versus* 99.2 (93.9–104.5) and 98.0 (81.4–114.6) h, respectively; 5% critical value, Tukey-Kramer method for unplanned comparisons among means]. There was no significant difference in development time among the Maine eggs. Mean egg development times were very similar to those previously obtained at 20°C (97.3 and 70.4 h for SR and MD locales, respectively; see Table II, Lonsdale and Levinton, 1985a).

Egg size

Mean egg volume of a clutch (μm^3) was not significantly affected by total incubation time (0 to 72 h for MD and 0 to 96 h for SR and SHP), as the regression equations were not significant for several models tested (*e.g.*, linear, $P > 0.5$, 0.1, and 0.1, respectively). Thus, the incubation time series data were pooled by collection locale. Both locale and family within locale influenced egg volume (Nested ANOVA using $\log_{10}$ transformed data sets; df = 2,217, F = 44.94, $P < 0.001$ and df = 41,217, F = 5.99, $P < 0.001$, respectively; Fig. 1). MD females produced significantly smaller eggs than either SR or SHP females [6.18 (5.75–6.64) × 10^4 μm^3 *versus* 8.37 (7.91–8.86) and 8.09 (7.59–8.62) × 10^4 μm^3, respectively] and there was no difference between the latter two locales (5% critical value, Tukey-Kramer method).

The mean dry mass of females was 8.2 (7.2–9.2), 9.1 (8.3–9.9), and 7.4 (6.7–8.1) μg for MD, SR, and SHP, respectively. Differences in dry mass did not account for the locale effects on egg volume (ANCOVA for dry mass

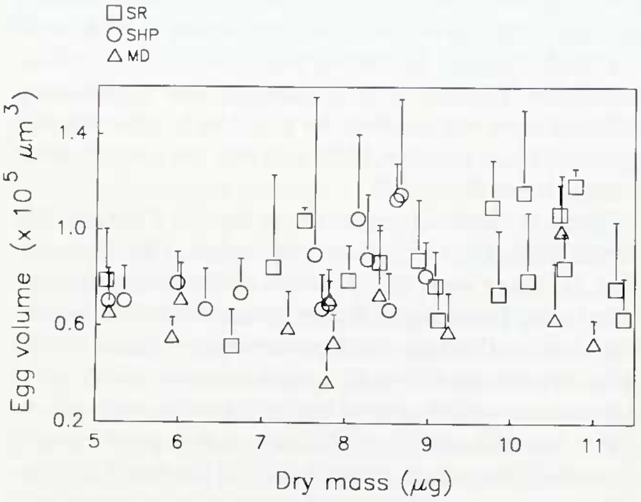

Figure 1. Mean egg volume (+95% confidence interval) and dry mass of females of *Scottolana canadensis* collected from three locales [Saco River (SR), Sheepscott River (SHP), and Patuxent River (MD)] and reared at 20°C and 2.5 × 10^5 cells ml^{-1}.

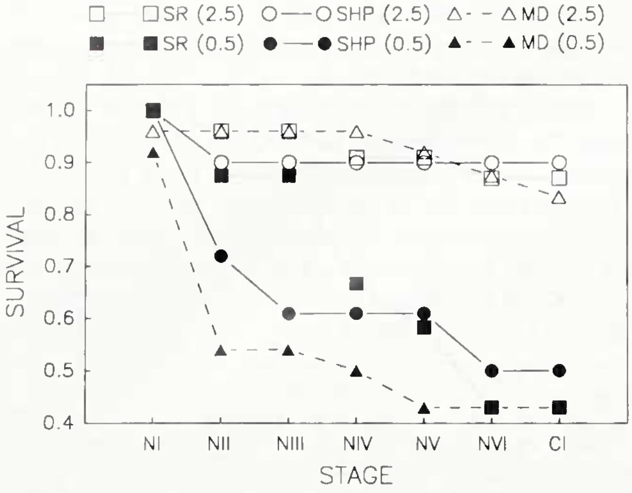

Figure 2. Survival of *Scottolana canadensis* at each naupliar stage when reared at 20°C and at concentrations of 2.5 and 0.5 × 10^5 cells ml^{-1}.

adjusted volumes; df = 2,257, F = 21.24, $P < 0.001$, Fig. 1).

Naupliar newborn size, survivorship, and growth rate

The length (μm) of newborn nauplii was influenced by both locale and family within locale effects (Nested ANOVA using $\log_{10}$ transformed data sets; df = 2,78, F = 11.45, $P < 0.001$ and df = 15,78, F = 2.46, $P < 0.01$, respectively). MD newborns were significantly smaller than those from SHP and SR, and there was no difference among the latter two groups [91 (90–92) μm *versus* 99 (98–101) and 100 (99–101) μm length, respectively; 5% critical value, Tukey-Kramer method]. Similarly, locale and family within locale were significant factors affecting maximum head width (Nested ANOVA; df = 2,78, F = 42.96, $P < 0.001$ and df = 15,78, F = 4.11, $P < 0.001$, respectively). All locales were significantly different from one another [54 (53–55), 57 (56–59), and 61 (60–63) μm for MD, SHP, and SR; 5% critical value, Tukey-Kramer method].

Naupliar survival was poorer at the low food concentration than at the high food concentration for all locales (Fig. 2). There were no significant differences among locales in the proportion of nauplii surviving through the NV stage at the high food concentration (paired t-test using arcsine transformed proportions of survivors at each stage; df = 4, $t_s = 0.913$, $P > 0.4$ in a comparison of SHP *versus* SR, and $t_s = 0.112$, $P > 0.9$ in a comparison of pooled Maine data *versus* MD). At the low food concentration, no significant difference in survivorship was found among SR and SHP nauplii ($t_s = 0.755$, $P > 0.5$), but the survival of MD nauplii was significantly less than that of Maine nauplii ($t_s = 3.733$, $P < 0.05$). The survival advantage of Maine nauplii was lost during the NVI stage (Fig. 2).

Molt lengths of MD nauplii were in general shorter than those of Maine *S. canadensis* (Fig. 3). The molt length of CI copepodites was affected by collection locale and by food concentration (Two-way ANOVA using $\log_{10}$ transformed data sets; df = 2,74, F = 6.73, $P < 0.01$ and df = 1,74, F = 25.50, $P < 0.0001$, respectively; Table II. The influence of family within locale was not investigated because some families had no CI survivors at the low food concentration). The low food concentration resulted in smaller copepodites, and those from MD were smaller at the high food concentration than either SR or SHP copepodites (5% critical value, Tukey-Kramer method). At the low food concentration, the molt lengths of the copepodites collected from different locales were not significantly different.

The low food concentration also resulted in a longer total development time (h) to CI (Two-way ANOVA us-

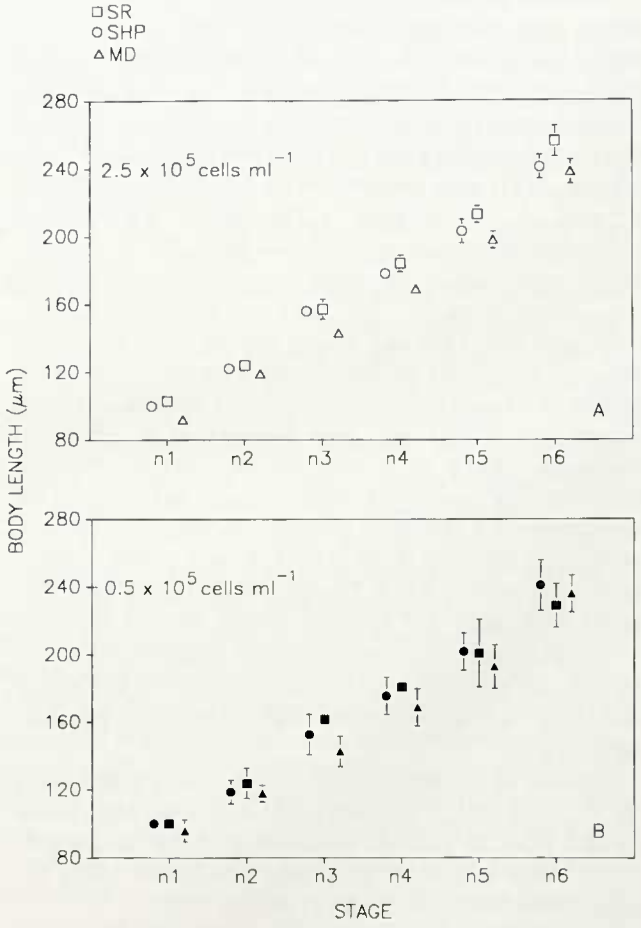

Figure 3. Mean molt lengths (±95% confidence interval) of *Scottolana canadensis* nauplii collected from three locales [Saco River (SR), Sheepscott River (SHP), and Patuxent River (MD)] and reared at 20°C and either 2.5 or 0.5 × 10^5 cells ml^{-1}. Missing confidence intervals are smaller than the height of the symbol.

ing $\log_{10}$ transformed data sets; df = 1,79, F = 62.00, $P < 0.0001$; Table II) and although MD nauplii had a shorter mean development time at both food concentrations, locale variation was marginally not significant (df = 2,79, F = 3.03, $P = 0.054$).

Regressions of molt length (log transformed) *versus* total development time were significant for all of the growth rate studies ($P < 0.001$ for all regressions; Fig. 4). At the high food concentration, the regression coefficient for MD was significantly higher than either SR or SHP, and the latter two were not different from one another (Tukey-Kramer method for unplanned comparisons among a set of regression coefficients using 5% critical values). However, there were no significant locale differences among regression coefficients at the low food concentration, and all those coefficients were significantly lower than the ones obtained under the high food concentration.

Discussion

Our results did not support the "nauplius development time restriction" hypothesis (Lonsdale and Levinton, 1985a). Total development time to CI was not less for SR nauplii than for those from either MD or SHP at either food concentration. *Scottolana* from the Saco River estuary were also similar to copepods from the Sheepscott River estuary in other life-history traits, such as egg development time, egg volume, and newborn size. This research, therefore, provides more evidence for genetically based, latitudinal differences in life-history traits of *S. canadensis* (between Maine and Maryland).

The adaptive significance of latitudinally related variation in newborn size in *S. canadensis* remains unclear and may, in fact, be due to genetic drift of isolated populations (see Hines, 1986, and Slatkin, 1987, for general reviews). On the other hand, the attainment of a larger newborn size appears to encumber a fitness cost in terms of prolongation of embryogenesis. In turn, this cost may be offset by the survival advantages of a larger body size. For example, the predation rate of the adult copepod *Acartia tonsa* on copepod nauplii was inversely related to naupliar body size (when NI-III and NIV-VI were compared), although prey swimming ability and behavior also may have been important (Lonsdale *et al.*, 1979; Tackx and Polk, 1982). In MD, however, invertebrate predation is an important regulator of *S. canadensis* population growth (Lonsdale, 1981), and thus, the laboratory differences in naupliar size *per se* are not likely a reflection of habitat variation in this selective pressure.

We suggest an additional fitness advantage of larger body size. A preliminary examination of eggs preserved within 4 h of mean hatching time (72 h for MD copepods and 96 h for Maine) revealed that the total lipid staining area (μm^2 using Oil Red O; Gallager and Mann, 1981) of the eggs was not different among locales [62 (11–113), 89 (19–159), and 112 (−32–256) μm^2 for MD, SR, and SHP, respectively)]. Although these area measurements are not an optimal estimate of lipid content (Gallager and Mann, 1986), they do suggest that variation in maternal reserves did not contribute to the survival differences of the nauplii under low food stress. Thus, there may have been size-related differences in naupliar feeding efficiency (energy ingested–energy expended; Hall *et al.*, 1976; Sebens, 1982). Larger body size may result in a greater filtering capacity (per animal), or greater difference between weight-specific energy acquisition and expenditure, as compared to smaller forms (Hall *et al.*, 1976; Gliwicz, 1990). The survival patterns of *S. canadensis* may indicate that under the high food concentration, differences were not found because the energetic demands of growth were met for both MD and Maine nauplii. But under the low food concentration, the energetic impact of lower feeding efficiency resulted in higher mortality of the MD copepods. The survival advantage of Maine nauplii was lost during the NVI stage, perhaps for several reasons. First, at NVI, the molt lengths of the Maine and MD nauplii were similar (Fig. 3B) and thus, there would be no size-related advantage. Second, the metamorphosis of *S. canadensis* nauplii to copepodites was associated with the highest stage-specific growth rate ($\mu m\ h^{-1}$) compared to all other stages of nauplii and required substantial morphological change.

In previous studies, the mean growth rate (μg dry mass d^{-1}) of SR and MD females was usually not different, particularily at 20°C, nor were the components of growth rate (*i.e.*, adult dry mass and total time from NI to the adult molt; Lonsdale and Levinton, 1985b, 1986). Yet growth rate differences during the naupliar stages have been shown in this study. However, in this study, the growth rates of SR and MD female copepodites (CI to adult molt) were similar [$\log Y = 2.35 + 0.00187 (\pm 0.00023) X$ and $\log Y = 2.32 + 0.00195 (\pm 0.00036) X$ where $Y = \mu m$ and $X = h$, respectively and at the high food concentration; unpubl.]. This result may help explain the lack of concordance between mean growth rate to adult and naupliar growth rate with regard to the influence of collection locale.

The egg volumes determined in this study were ~25–30% less than those reported by Lonsdale and Levinton (1985a) for both the Maine and MD locales. This discrepancy may be due to differences in the preservation process; in the previous study copepods were not narcotized with $MgCl_2$ prior to preservation in formalin. Initial estimates of egg volumes of Maine *S. canadensis* individuals collected from the field, and which were not narcotized prior to formalin preservation, were more similar to those reported by Lonsdale and Levinton

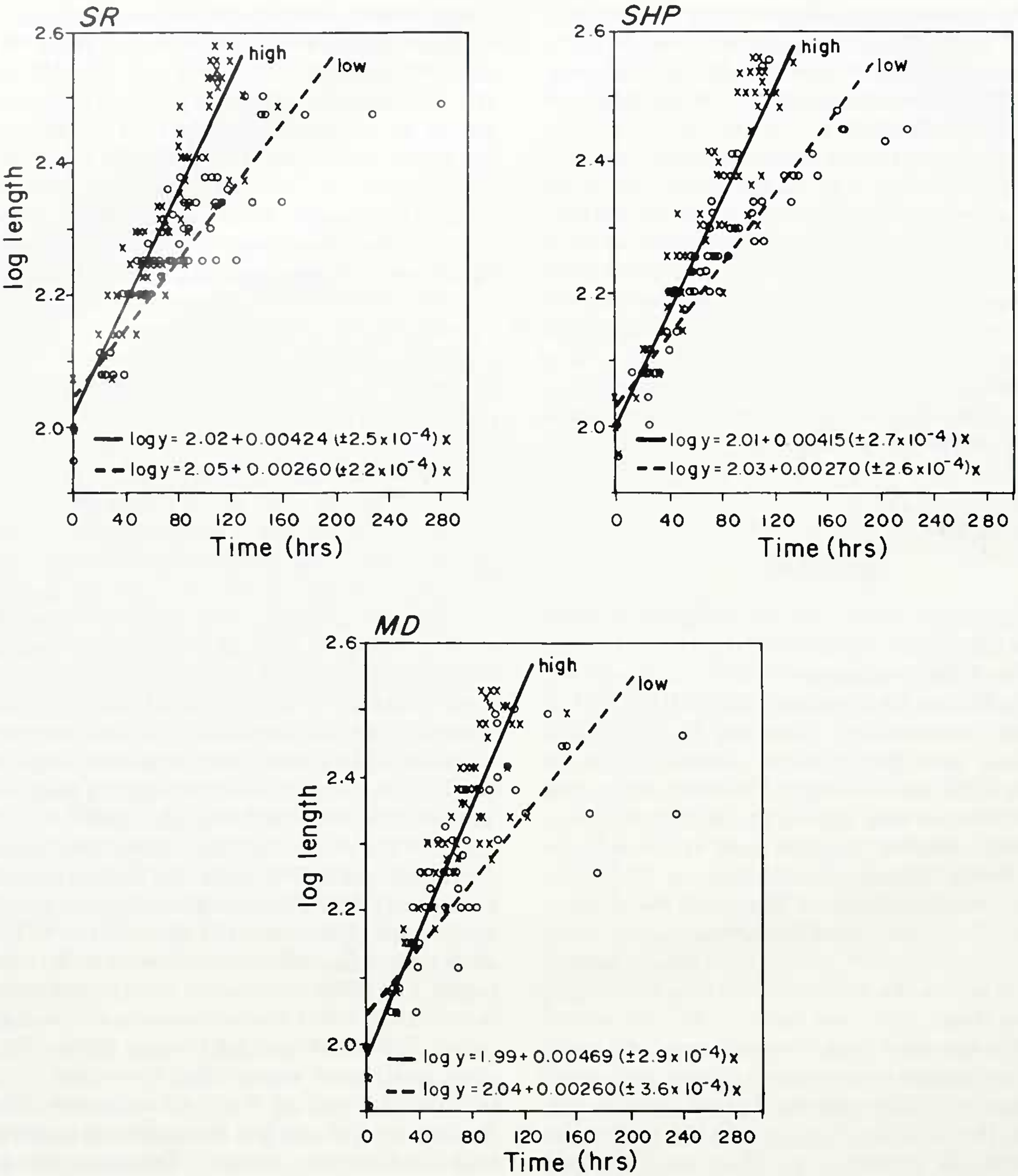

Figure 4. Growth rate ($\log_{10}$ μm h^{-1}) of *Scottolana canadensis* nauplii collected from three locales [Saco River (SR), Sheepscott River (SHP), and Patuxent River (MD)] and reared at 20°C and either 2.5 × 10^5 cells ml^{-1} (solid lines and x data points) or 0.5 × 10^5 cells ml^{-1} (dashed lines and ○ data points). The 95% confidence interval of the slope is provided in the regression equation.

(1985a). The average egg volume from field-collected females ranged from 1.17 to 1.40 × 10^5 μm^3 when temperatures ranged from about 13 to 18°C (unpubl.).

An alternate hypothesis of the "nauplius development time restriction hypothesis" to explain differences in newborn size of *S. canadensis* is that variation in primary productivity between latitudinally separated locales may be significant (Lonsdale and Levinton, 1985a). In Chesapeake Bay during the spring bloom, the chlorophyll concentration exceeded 40 mg m^{-3} in the euphotic zone and 29–48 mg m^{-3} over the entire water column [Malone *et al.*, 1988. Data were converted from mg m^{-2} by using the average euphotic depth (5 m) or range of channel depths (25–42 m), respectively]. In the Damariscotta River estuary (just northeast of the Sheepscott River estuary), spring bloom chlorophyll concentrations occurred in late February and early to mid-March with a maximum value of 10 $\mu g\,l^{-1}$ (Townsend, 1984). During May and June when *S. canadensis* have been found in high abundance in Maine (Lonsdale and Levinton, 1985a), the chlorophyll values ranged from 4 to 5 $\mu g\,l^{-1}$ (Townsend, 1984). Although chlorophyll concentration *per se* is not always an adequate indicator of food availability (Bellatoni and Peterson, 1987), we suggest that the variation in newborn size found among *S. canadensis* nauplii may reflect differences in food availability between the Maine and MD habitats.

At present, biochemical measurements of genetic variation among and within *S. canadensis* populations are lacking. Despite common-rearing, some of the variation found may not be related to genetic differences, but to other irreversible, non-genetic effects (*e.g.*, maternal effects due to the handling history of individual females within a locale). Ecological studies of the energy demands of copepod nauplii during development and the coupling of primary production to patterns of copepod reproduction and survival in the field would further our understanding of the evolution of life-history strategies of marine invertebrates.

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Consequences of the Calcite Skeletons of Planktonic Echinoderm Larvae for Orientation, Swimming, and Shape

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Abstract. How the echinoderm larval skeleton is used for support of larval arms, passive orientation, and swimming was examined by experimentally removing the skeletons of plutei and by comparing feeding larvae from four echinoderm classes. All four types of echinoderm larvae oriented with their anterior ends upward in still water, but removing the skeletons of both live and dead four-armed echinoplutei demonstrated that their skeletons enhanced passive vertical orientation with their anterior ends upward. In comparisons of dead four-armed echinoplutei with and without skeletons, the skeleton contributed more than half of the excess density and sinking speed. In comparisons of all four types of feeding echinoderm larvae, larvae with a greater volume of skeleton and a smaller volume of tissues and body cavity were densest. The calculated work necessary to prevent the plutei from sinking was much less than 1% of the total aerobic energy expenditure. Thus calcite skeletons are not essential for passive vertical orientation by echinoderm larvae but enhance it, and the increased density and sinking rates impose little energetic cost in locomotion.

The evolution of larval skeletons may have been influenced by the benefits of passive orientation and by the low costs of swimming with a skeleton. Whatever the primary function of skeletons, the position and form of skeletal elements is influenced by the functional requirement for higher mass posteriorly for passive orientation. Features that enhance passive vertical orientation include posterior ossicles and skeletal rods, posterior thickening of skeletal rods, and formation of juvenile parts near the posterior ends of larvae.

Introduction

Some echinoderm larvae (echinoplutei, ophioplutei) have a skeleton with calcite rods that support projecting arms, but others (auriculariae, bipinnariae) do not. It is peculiar that any small gelatinous planktonic animals should have an internal mineral skeleton for body support, but a supporting function for some internal skeletal elements is obvious. Calcite rods support the soft parts of a pluteus against the contraction of the muscles that dilate its mouth during the rejection of large particles from its oral cavity (Strathmann, 1971). Calcite rods provide the attachment and leverage for muscles that spread the arms of some plutei, presumably in defense (Mortensen, 1921, 1938). Other supporting functions are also likely. Gustafson and Wolpert (1961) suggested that the arm rods of plutei support an epidermis under tension. Emlet (1982, 1983) measured the stiffness of calcite skeletal rods of plutei and analyzed the forces that swimming imposes on projecting arms. He concluded that fenestrated skeletal rods are stiffer than needed to support the arms against forces generated in ciliary swimming and suggested that the stiffness may protect the larvae against predators.

Some calcite skeletal elements, however, do not support soft parts. Some, such as the posterior ossicle in the auricularia, are only solitary lumps that have no imaginable supporting function. Also, the posterior ends of the body rods of four-armed echinoplutei are much thicker

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than the arm rods but appear to be subject to no greater forces and are resorbed at later stages. Runnström (1918) and Pennington and Emlet (1986) suggested that the body skeleton of the echinopluteus is a counterweight that orients the larva with its arms upward. Echinoderm larvae have no known statocyst or organ for sensing gravity, yet all feeding larvae tend to swim with their anterior ends upward. Mechanisms enhancing passive orientation may help them maintain a favorable position in the water column.

We are concerned here with the hypothesis that passive vertical orientation has been an important functional requirement in the evolution of echinoderm larval body plans and especially of larval skeletons. That requirements for passive vertical orientation might constrain larval body plans or entail ecological consequences has been little appreciated or studied.

If the skeleton contributes substantially to passive vertical orientation, it also contributes to the excess density of the larva. Excess density is the difference between the density of an object and the density of seawater. Excess mass is the excess density of an object times its volume. The larval center of gravity is the center of its excess mass, and its center of buoyancy is the center of its volume. The weight of a body acts at the center of gravity, and the upthrust from weight of fluid displaced acts at the center of buoyancy (Alexander, 1968). If the skeleton orients the larva passively with anterior end upward, then the larva's center of gravity, as controlled by the skeleton and other parts, must be posterior to the center of buoyancy.

If the density of the soft parts (tissues and body cavities) of a larva was equal to that of seawater, then the effect of the skeleton on passive vertical orientation might be deduced from anatomical measurements of skeletal components alone. However, some soft parts are denser than seawater; some may be less dense than seawater; and the volume of soft parts greatly exceeds the volume of skeleton. For example, Pennington and Emlet (1986) found that four- and six-armed echinoplutei of *Dendraster excentricus* were denser than eight-armed plutei or even newly metamorphosed juveniles and suggested that accumulation of lipids in the gut (Burke, 1981) might account for the lower density at advanced stages. In addition, the density of the contents of the primary body cavity may differ from the density of cells, and the primary body cavity can be small or extensive, depending on the stage and type of larva. Thus passive vertical orientation by an appropriate distribution of density along the body axis would constrain the distribution of cells, body cavities, and skeleton. Demonstrating a role of the skeleton in passive orientation therefore requires more than anatomical measurements.

In this study we examined the calcite skeletons of planktotrophic echinoderm larvae to identify those functional consequences of skeletal size and shape that may have influenced the evolution of these larval forms. To examine the role of calcite skeletons in passive vertical orientation, we (1) experimentally removed skeletons from echinoplutei and (2) compared types of planktotrophic echinoderm larvae that differ in skeletal development. These studies also addressed two related topics: the role of the calcite skeletons in the maintenance of body shape, and the contribution of calcite skeletons to excess density and sinking rate.

Materials and Methods

Rearing

The methods of obtaining gametes, fertilizing ova, and maintaining embryos and larvae are those in Strathmann (1987). Most larvae were reared in 1.5 to 3 liter cultures in mechanically stirred jars. Some cultures of 0- to 4-day-old larvae were kept in smaller containers and not stirred. Rearing temperatures were about 10–14°C at the Friday Harbor Laboratories on San Juan Island and 23–27°C at the Kewalo Marine Laboratory on Oahu, within a few degrees centigrade of the local sea temperatures.

At the Kewalo Marine Laboratory, larvae were fed *Rhodomonas lens* (as described in Leahy, 1986), occasionally supplemented with *Dunaliella tertiolecta*. These larvae were the echinoplutei of *Echinometra mathaei* (Blainville), *Tripneustes gratilla* (Linnaeus), and *Colobocentrotus atratus* (Linnaeus).

Most larvae at the Friday Harbor Laboratories were fed natural phytoplankton supplemented with *Dunaliella tertiolecta* and occasionally also *Thalassiosira weissflogii* or *Isochrysis galbana*. These larvae were the auriculariae of *Parastichopus californicus* (Stimpson), the bipinnariae of *Asterina miniata* (Brandt) and *Evasterias troschelii* (Stimpson), the ophioplutei of *Ophiura sarsi* Lütken and *Ophiopholis aculeata* (Linnaeus), and the echinoplutei of *Strongylocentrotus droebachiensis* (O. F. Müller), *S. pallidus* (G. O. Sars), *S. purpuratus* (Stimpson), and *Dendraster excentricus* (Eschscholtz).

Killing

Dead larvae were required for several experiments. At Kewalo Marine Laboratory larvae were killed with 2% formalin buffered with calcium carbonate in seawater. At the Friday Harbor Laboratories larvae were killed with sodium cyanide (about 0.005 to 0.001 M) in seawater.

Decalcification

Different methods were used to decalcify dead or live echinoplutei. The skeletons of dead, formalin-fixed larvae were removed by placing the larvae in a 1:1 mixture of seawater and EDTA-saturated seawater for 2–10 min. Complete decalcification was verified by viewing larvae under a dissection microscope with crossed polarizing filters: any remaining skeleton produced a bright glow.

The skeletons of live echinoplutei were removed by the method of Pennington and Hadfield (1989). Batches of several hundred early four-armed plutei (3–5 days old) were pipetted into synthetic seawater (MBL recipe; Cavanaugh, 1956) buffered at pH 5.5 or 6.0 with a final concentration of 0.03 *M* MES (2-[N-Morpholino]ethanesulfonic acid). Although the rate of decalcification varied with the species, number, and stage of larvae, the plutei usually decalcified in 2 to 5 h; they were then removed to natural seawater. Because this treatment temporarily suppressed motility, the larvae were allowed to recover overnight before behavioral observations were made. Decalcified echinoplutei were maintained in groups of 1–50 larvae in 20-ml finger-bowls and fed *R. lens.*

Orientation, density, and sinking

Orientations and relative densities were compared for dead larvae sinking in density gradients. At Friday Harbor at least three and at Kewalo at least two larvae of each type were pipetted into the top of cuvettes about 3 cm tall by 1 cm wide and deep or in some cases about 10 cm high by 1.3 cm wide and deep. The cuvettes contained seawater at the top and a denser fluid at the bottom; partial mixing of the two fluids produced a density gradient. Except where noted, the denser fluid was Percoll in a solution of NaCl isosmotic with seawater. Percoll is a colloidal suspension of silica particles coated with polyvinylpyrrolidine and was obtained from Pharmacia. Some observations were repeated with sucrose isosmotic with seawater as the denser solution. Resting orientation at neutral buoyancy was checked by reorienting the larva with a glass needle and then waiting several minutes to see if it would return to its original position. (The needle was swept past the larva so that the return stroke did not rotate the larva back to its original position.)

The densities of echinoplutei with and without skeletons were estimated in three experiments. Dead four-armed plutei of *T. gratilla* (4–5 days old) were suspended in seawater layered over a series of Percoll dilutions of decreasing specific gravity but isosmotic with seawater. If larvae floated above the Percoll for 30 min, their density was recorded as less than that of the Percoll solution; if they sank to the bottom, their density was greater than that of the Percoll solution. The densities of the Percoll dilutions were obtained by weighing known volumes. Isosmotic Percoll solutions were used instead of the sucrose solutions of Pennington and Emlet (1986), who used sucrose solutions without adjusting osmolarity. When the Percoll method was compared with sucrose solutions of unadjusted osmolarity for both normal and decalcified plutei of *T. gratilla,* higher estimates of larval density were produced with the sucrose.

To compare the orientation and sinking rates of echinoplutei with and without skeletons, we used four-armed plutei of *T. gratilla* (3 and 5 days old). Observations of dead larvae with and without skeletons were made in cuvettes, as described above. In seawater without a density gradient, currents were eliminated by minimizing air currents, covering the cuvettes, using indirect backlighting, and equilibrating air and water temperatures. Motionless suspended particles indicated an absence of currents. These observations may underestimate sinking rates because of wall effects in the small cuvettes. Larger cuvettes in water baths were impractical because plutei without skeletons were difficult to see. We measured the sinking rates of larvae that were away from the walls. The observations were adequate to demonstrate an effect of the skeleton and to provide a check on other measurements of the contribution of the skeleton to the density of the larval body.

Stability of vertical orientation was compared for echinoplutei with and without skeletons. Convection currents in the cuvettes were enhanced by shining a lamp on the cuvette wall so that the current rose on one side and descended on the other. Dead four-armed echinoplutei of *T. gratilla* (3 days old), either with or without skeletons, were pipetted into the top of the descending current. The vertical orientations of the first larvae carried upward past mid-depth in the ascending current were scored as (1) arms-up, (2) arms-down, or (3) horizontal.

Orientation during swimming was compared for live echinoplutei with and without skeletons. Decalcified and normal plutei of *T. gratilla* and *C. atratus* were videotaped. Upward swimming indicated orientation to gravity. Swimming in other directions or tumbling indicated deficient orientation to gravity.

Results

Effects of experimental removal of skeletons

Shape. Decalcified dead echinoplutei retained the pluteus form despite the complete loss of skeleton (Fig. 1).

Decalcified live echinoplutei of *E. mathaei, T. gratilla,* and *C. atratus* were maintained for 74, 48, and 19 days, respectively. The larval shape changed during this time. Larvae without skeletons initially retained some features of the pluteus shape (Fig. 2A, B). Larval arms were shorter than arms of control plutei with skeletons (Fig. 2C) but were still present (Fig. 2B). Arms without a sup-

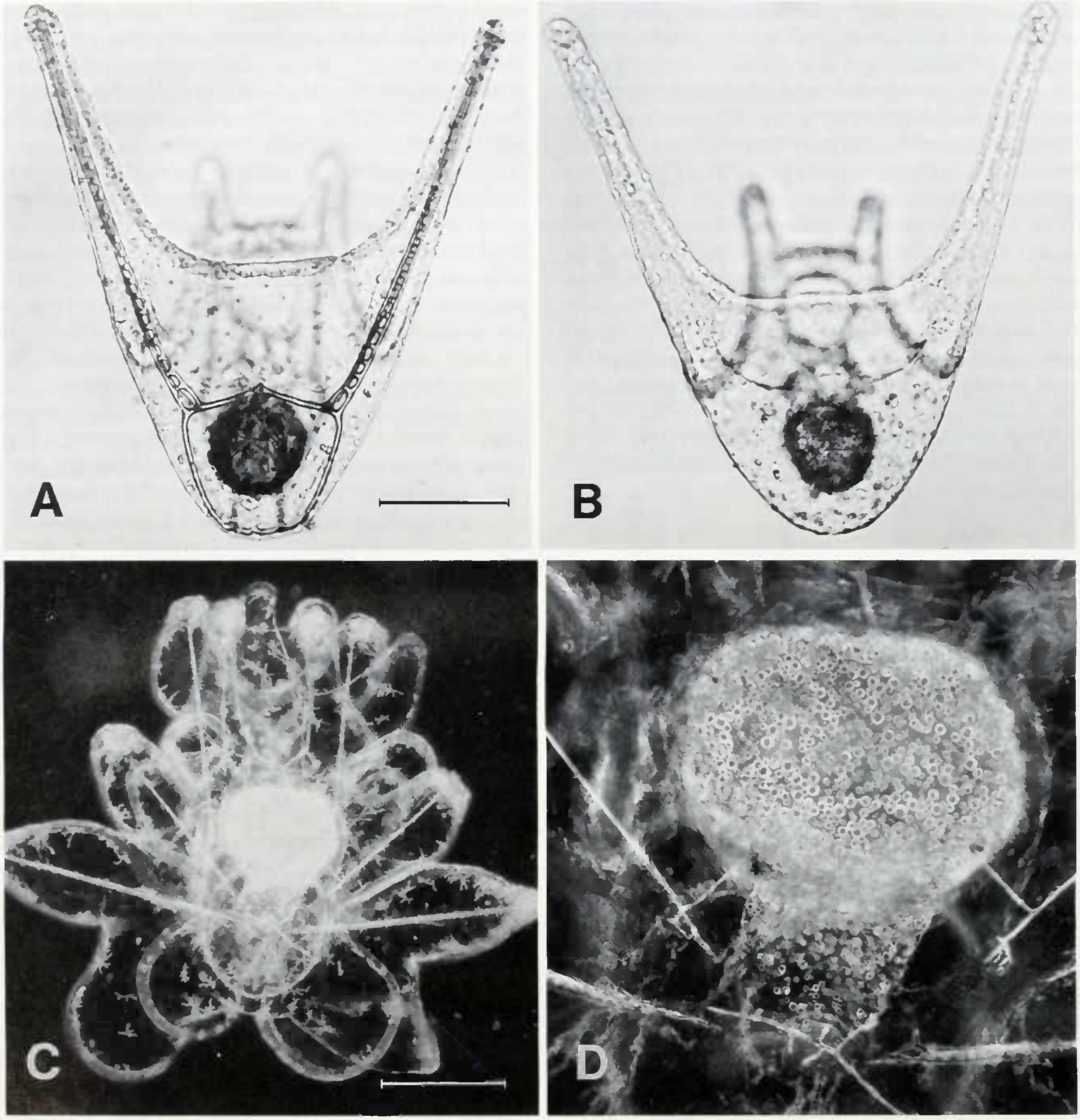

Figure 1. Echinoplutei of *Tripneustes gratilla*, 6 days old: (A) normal control larva with 100 μm scale bar and (B) larva killed with formalin and decalcified with EDTA, same scale. Late eight-armed echinopluteus of *Tripneustes gratilla* with echinus rudiment: (C) whole larvae with 300 μm scale bar and (D) stomach with refractile droplets that are absent in the stomach wall of larvae at earlier stages (3 times the magnification of C).

porting skeleton did not bend noticeably during swimming. The esophagus was initially constricted in *T. gratilla*, but later opened. The mouth, stomach, and intestine appeared normal in all three species, and the larvae without skeletons captured and ingested phytoplankton cells. Some plutei did not resecrete a skeleton, and they completely resorbed their arms over the course of several days. These larvae became bowl or saucer shaped but retained distinct oral hoods and concave circumoral fields (Fig. 2D). The ciliated band persisted as the rim of the bowl. However, most larvae resecreted abnormal skeletons (Fig. 2E, F). Their arms developed to various

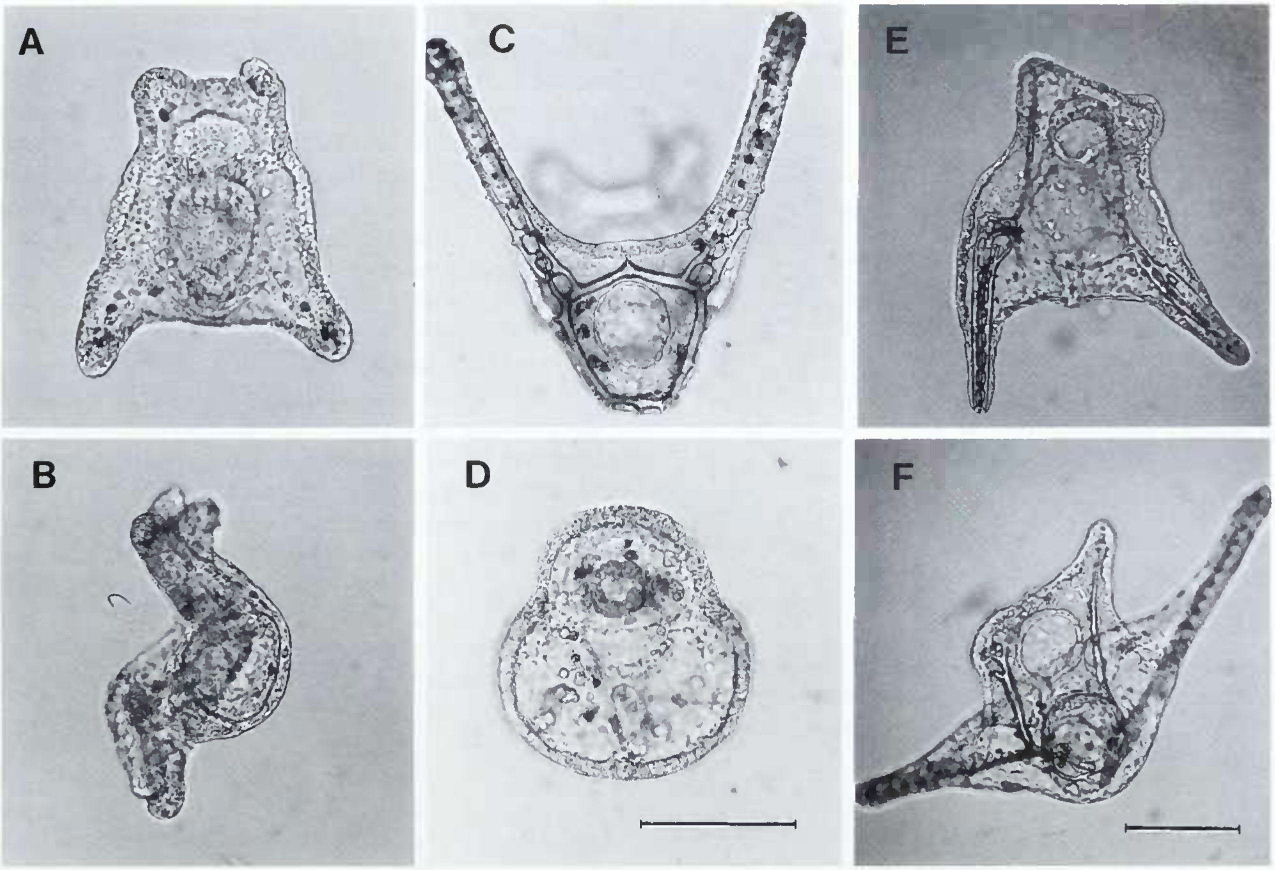

Figure 2. Live four-armed echinoplutei of *Colobocentrotus atratus*: (A) oral and (B) lateral views of recently decalcified plutei, (C) ventral view of normal control larva of same age as larvae in A and B, (D) oral view of larva that had been decalcified several days previously and did not regrow a skeleton, (E) and (F) oral views of plutei that had been decalcified several days previously and did regrow a skeleton.

lengths and in odd directions, and their body skeletons were equally abnormal. Most developed four arms, the correct number, though some had missing or extra arms. In *C. atratus* and *T. gratilla*, the postoral arm rods were usually fenestrated as in normal control larvae. No decalcified larvae developed to six-armed or later stages, but conditions in 20-ml culture dishes were not favorable.

Skeletons in echinoplutei appeared to play a small role in immediate mechanical support and a greater role in long-term maintenance and development of larval form.

Passive orientation. In still seawater, almost all four-armed echinoplutei of *T. gratilla* with and without skeletons oriented passively with their arms upward. Nearly 100% of dead larvae with skeletons sank with arms upward, and about 80% of those without skeletons sank with arms upward. However, removal of the skeleton did change the stability in the arms upward orientation. In a cuvette with 100% seawater and side illumination, there were convection currents and velocity gradients; dead echinoplutei with skeletons were oriented arms upward, but dead echinoplutei without skeletons tumbled. Of larvae at mid-depth in the ascending current, 100% of those with skeletons were arms-upward (n = 30) and only 28% of those without skeletons were arms-upward with the remainder arms-downward or horizontal (n = 40). We expected that with random orientation about 25% of larvae should have been arms upward. The plutei without skeletons did not differ significantly from this expectation (Chi-square, $P > 0.9$), while the plutei with skeletons were clearly non-random in their orientation ($P < 0.005$). Removal of the skeleton greatly decreased passive stability.

Orientation of living echinoplutei. The vertical orientation of live four-armed echinoplutei of *C. atratus*, *E. mathaei*, and *T. gratilla* was compared with and without skeletons. Most plutei with skeletons swam with their arms upward. Forward swimming produced upwards movement. The arms-upward orientation was less pronounced after decalcification. Plutei of *C. atratus* were the most vigorous after decalcification. Almost all of these plutei tumbled in an anterior-posterior direction with little or no net movement. A few decalcified plutei

Table I

Ranking of density of some Hawaiian echinoplutei from least dense to most dense with larvae of nearly equal density lumped at the same rank

Rank	Species	Stage	Reorientation
1	*Tripneustes gratilla*	four-armed decalcified	*
1	*Tripneustes gratilla*	eight-armed with skeleton	*
2	*Echinometra mathaei*	eight-armed with skeleton	*
3	*Tripneustes gratilla*	four-armed with skeleton	*
3	*Colobocentrotus atratus*	eight-armed with skeleton	*

* = Those that reoriented as they sank toward neutral buoyancy.

did swim forward without tumbling, though they swam downwards as well as upwards with no clearly discernible orientation to gravity. When disturbed, larvae with and without skeletons stopped or swam backwards. Results with *E. mathaei* and *T. gratilla* were similar, though decalcified larvae of these species swam less vigorously.

Density. When the skeletons of four-armed (4 and 5 day old) echinoplutei of *T. gratilla* were removed, the density of the larval body decreased. The density of larvae with skeletons was estimated to be about 1.06 g/ml, because all dead larvae with skeletons sank through a Percoll solution of density 1.042 g/ml, and all floated above a solution of density greater than 1.078 g/ml. More than half (*ca.* 70%) sank in a solution of density 1.054 g/ml. The density of larvae without skeletons was estimated to be about 1.04 g/ml because all dead larvae without skeletons sank through a solution of density 1.037 g/ml and all floated in a solution of density 1.042 g/ml.

In a density gradient, the terminal depth of four-armed plutei of *T. gratilla* without skeletons was less than the depth of control plutei with skeletons (Table I). This result again indicates that the skeleton increases the density of the larval body.

Sinking speed. Removal of skeletons of four-armed echinoplutei of *T. gratilla* decreased their sinking rates in seawater. When 3 days old, echinoplutei with skeletons sank at 0.29 mm/s (S.D. = 0.076 mm/s, n = 10), and plutei without skeletons sank at 0.052 mm/s (S.D. = 0.022 mm/s, n = 10). When 5 days old, echinoplutei with skeletons sank at 0.44 mm/s (S.D. = 0.21 mm/s, n = 10), and plutei without skeletons sank at 0.052 mm/s (S.D. = 0.018 mm/s, n = 10). In both comparisons the sinking speeds were significantly less for plutei without skeletons (*t*-test, $P < 0.001$).

Comparisons of larval forms and stages

Passive orientation. Echinoderm larvae of four classes passively oriented with their anterior ends upward while sinking in seawater at the top of Percoll gradients. The dead larvae that sank with their anterior ends upward were auriculariae of *P. californicus*, bipinnariae of *A. miniata* and *E. troschelii*, ophioplutei of *O. aculeata* (four-armed) and *O. sarsi* (six-armed), and echinoplutei of *D. excentricus* (four- and eight-armed), *S. franciscanus* (four- and eight-armed), *S. pallidus* (four-, six-, and eight-armed), *S. purpuratus* (four-armed), *C. atratus* (eight-armed), and *T. gratilla* (four-armed). All were tested in gradients made with seawater as the less dense solution and with a mixture of Percoll and isosmotic NaCl as the denser solution. Many were also tested in gradients made with seawater and an isosmotic sucrose solution, with no difference in results. At the seawater end of the density gradient, sinking dead larvae reoriented with their anterior ends upward almost immediately after being turned anterior end downward. The two exceptions were eight-armed echinoplutei of *T. gratilla* and *E. mathaei*, which did not clearly orient with their arms upward while sinking into the top of a Percoll gradient.

Dead larvae of all four types also oriented anterior end upward when sinking in 100% seawater with no density gradient, except that the larvae were sometimes turned by the convection currents that developed under uncontrolled conditions in cuvettes lacking a density gradient.

Sinking was not required for passive orientation. Orientation was stable for larvae suspended at neutral buoyancy. However, some larval forms reversed their orientation as they sank into denser fluid because the distribution of excess mass was different in denser media. These results are addressed below, following the observations on density.

Density. Calcite skeletons affected larval density. In a density gradient, dead larvae with more skeleton in proportion to body volume came to rest in denser fluid (Tables I, II); larvae with little or no skeleton (auricularia and bipinnaria, Fig. 3A, B) or with the skeleton removed (pluteus of *T. gratilla*, Fig. 1B) were least dense. For comparisons among a range of larval forms, the larvae in Table II were ranked according to the proportion of body volume composed of calcite skeleton: 1 for the bipinnariae, with no skeleton; 2 for the auriculariae, with only a small ossicle; 3 for the plutei of *Strongylocentrotus* from four- to early eight-armed stages, with thin skeletal rods, an incomplete basket skeleton, and large spaces between skeleton and body wall; and 4 for the remaining plutei, with a relatively large skeleton because of thick rods, fenestrated rods, a more complete basket skeleton, or juvenile skeletal elements of the later rudiment. These ranks for skeleton as a proportion of body volume were correlated with the whole body densities in Table II (Spearman rank correlation 0.86, $P < 0.01$). These rank data comprise a broad range of forms of planktotrophic echinoderm larvae.

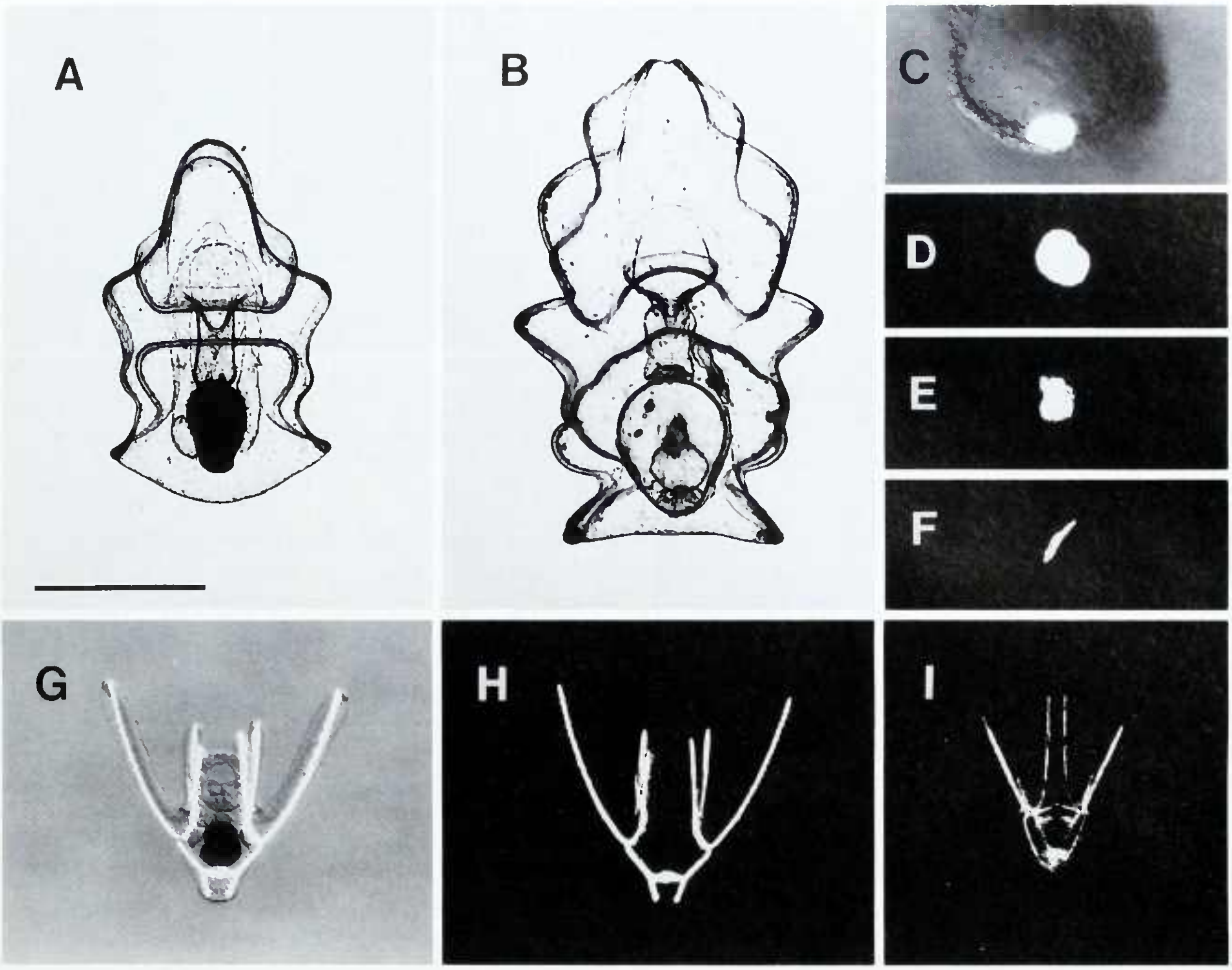

Figure 3. (A) Bipinnaria of *Asterina miniata*, (B) auricularia of *Parastichopus californicus*, (C, D, E) view of broad side and (F) view of narrow side of ossicles at the posterior, left corner of the body of *P. californicus*, (G) and (H) ophiopluteus of *Ophiura sarsi*, (I) echinopluteus of *Dendraster excentricus*. Calcite skeletons shown with polarizing filters partially crossed in (C) and (G) and fully crossed in (D, E, F, H, I). Scale bar is 400 μm in (A, B, G, H, I) and 160 μm in (C, D, E, F).

Among echinoplutei, the stage of development affected density. In a sample of 32 larvae of *S. franciscanus*, the eight-armed larvae with two or fewer pedicellariae and no juvenile spines or plates (Fig. 4E) were less dense than both the earlier stage four- to six-armed larvae (Fig. 4C, D) and the more advanced stage eight-armed larvae with three pedicellariae (Fig. 4F, G). (Juvenile plates and spines were forming in some of the larvae with three pedicellariae.) The differences in density among stages were statistically significant ($P < 0.001$, Kruskal-Wallis test). Eight-armed plutei were similarly less dense than four-armed plutei within the species *T. gratilla* (Table I, Fig. 1A, C) and *D. excentricus* (Table II). Very advanced eight-armed plutei were more dense than four-armed plutei in *S. pallidus* (Table II, Fig. 4H). The density changes during development were what one would have predicted from skeletal development. In early eight-armed stages of *Strongylocentrotus* species, the volume of skeleton relative to the volume of soft parts had decreased because the heavy ends of the body rods had disappeared and because the soft parts had grown more than the skeletal rods (Fig. 4). Also in *T. gratilla*, the plutei at the eight-armed stage (Fig. 1C) were much more fleshy than the plutei at the four-armed stage (Fig. 1A), and the density of eight-armed *T. gratilla* was almost as low as the density of the decalcified four-armed plutei (Table I). In these comparisons among stages, greater development of skeleton relative to soft parts was associated with greater density. However, Pennington and Emlet (1986) did not find an increase in density during rudiment development of larval *D. excentricus*. They suggested that lipids in cells of the gut (Burke, 1981)

Table II

Ranking of density of larvae from least dense to most dense with approximately equally dense larvae lumped together

Rank	Form	Species	Stage	Reorientation
1	auricularia	*Parastichopus californicus*		
1	bipinnaria	*Asterina miniata*		
2	echinopluteus	*Strongylocentrotus franciscanus*	eight-armed, small echinus rudiment	*
3	echinopluteus	*Strongylocentrotus franciscanus*	four-armed	*
3	echinopluteus	*Strongylocentrotus pallidus*	four-armed	*
4	echinopluteus	*Strongylocentrotus pallidus*	eight-armed, advanced echinus rudiment and postlarval plates and spines	*
5	echinopluteus	*Dendraster excentricus*	eight-armed	
6	echinopluteus	*Dendraster excentricus*	four-armed	
7	ophiopluteus	*Ophiopholis aculeata*	four-armed	

* = Those that reoriented as they sank toward neutral buoyancy.

might compensate for skeletal development at this stage. Refractile droplets appeared in the stomach cells of nearly competent plutei of *T. gratilla* (Fig. 1D), but we were not able to compare the specific gravities of all stages of *T. gratilla*.

Differences in whole body density among plutei of different species (Table II) were related to larval shape and skeletal development. The early four-armed pluteus of *S. purpuratus* (Fig. 4A, B) was denser than the early four-armed pluteus of *S. pallidus*. *S. purpuratus* develops from a smaller egg than does *S. pallidus*, and its pluteus begins with a larger skeleton relative to its body volume. Larvae of *S. purpuratus* converge on the form of other *Strongylocentrotus* larvae as they grow (Sinervo and McEdward, 1988), and presumably they converge in density at later stages also. Echinoplutei of *D. excentricus* have thick fenestrated arm rods and an extensive body skeleton (Fig. 3I) and, as expected, were denser than plutei of *Strongylocentrotus*. Four-armed ophioplutei of *O. aculeata* were the densest larvae examined (Table II), and ophioplutei in general have thick skeletal rods and a relatively small body cavity (Fig. 2G, H). (Plutei of *S. purpuratus* were not tested against plutei of *D. excentricus* or *O. aculeata* and therefore could not be included in Table II.) These comparisons indicate that the ratio of skeleton to whole body volume is a major determinant of density of the whole larval body.

Some differences in skeletons were too small to override other determinants of density of the larvae. The auriculariae of *P. californicus*, with their small posterior ossicles (Fig. 3B, C), and the bipinnariae of *A. miniata*, with no skeleton (Fig. 3A), sank to about the same depths in the density gradient. The ossicles of the auriculariae made no discernible difference in overall densities of the larval bodies. The auriculariae and bipinnariae had different lengths and body volumes, and this could have affected overall body density more than a small ossicle.

Similarly, in comparisons among auriculariae of *P. californicus*, density of the larval body was not discernibly affected by the presence or absence of a posterior ossicle, but it did vary with larval size. Size, shape, and location of posterior ossicles varies; so in a sample of 30 auriculariae from 2 pairs of parents, 7 auriculariae lacked any posterior ossicle, 20 had a posterior left ossicle, and 3 had an additional posterior right ossicle. In this sample there was no relation between density of the larval body (depth of neutral buoyancy in a Percoll gradient) and the occurrence of posterior ossicles. Most posterior ossicles were broad in two dimensions (commonly 30 to 50 μm) but thin in the third (about 10 μm) (Fig. 3D, E, F). Volumes of ossicles ranged from about 5,000 to 13,000 μm^3 (calculated as spheroids; sample of 4 larvae).

In the sample of 30 auriculariae, longer larvae were less dense than short larvae ($P < 0.02$, two-tailed rank-sum test, and Spearman rank correlation 0.59, $P < 0.001$). Longer auriculariae were also less dense in a comparison of 12 auriculariae, all from the same pair of parents and ranging in length from 650 to 870 μm ($P < 0.02$, two-tailed rank-sum test, and Spearman rank correlation 0.95, $P < 0.001$). Larger echinoderm larvae have larger primary body cavities but about the same thickness of body wall (McEdward, 1984), which is a single layer of cells. As an approximation, the volume of the body cavity should increase in proportion to the cube of body length and the volume of body wall in proportion to the square of length. The lower densities of longer auriculariae were therefore consistent with the hypothesis that their cells are denser than the contents of their primary body cavity, but other aspects of larval condition could have been confounded with size.

Effect of density of surrounding fluid on orientation

Passive orientation depends on the density of the surrounding fluid. All dead larvae passively oriented ante-

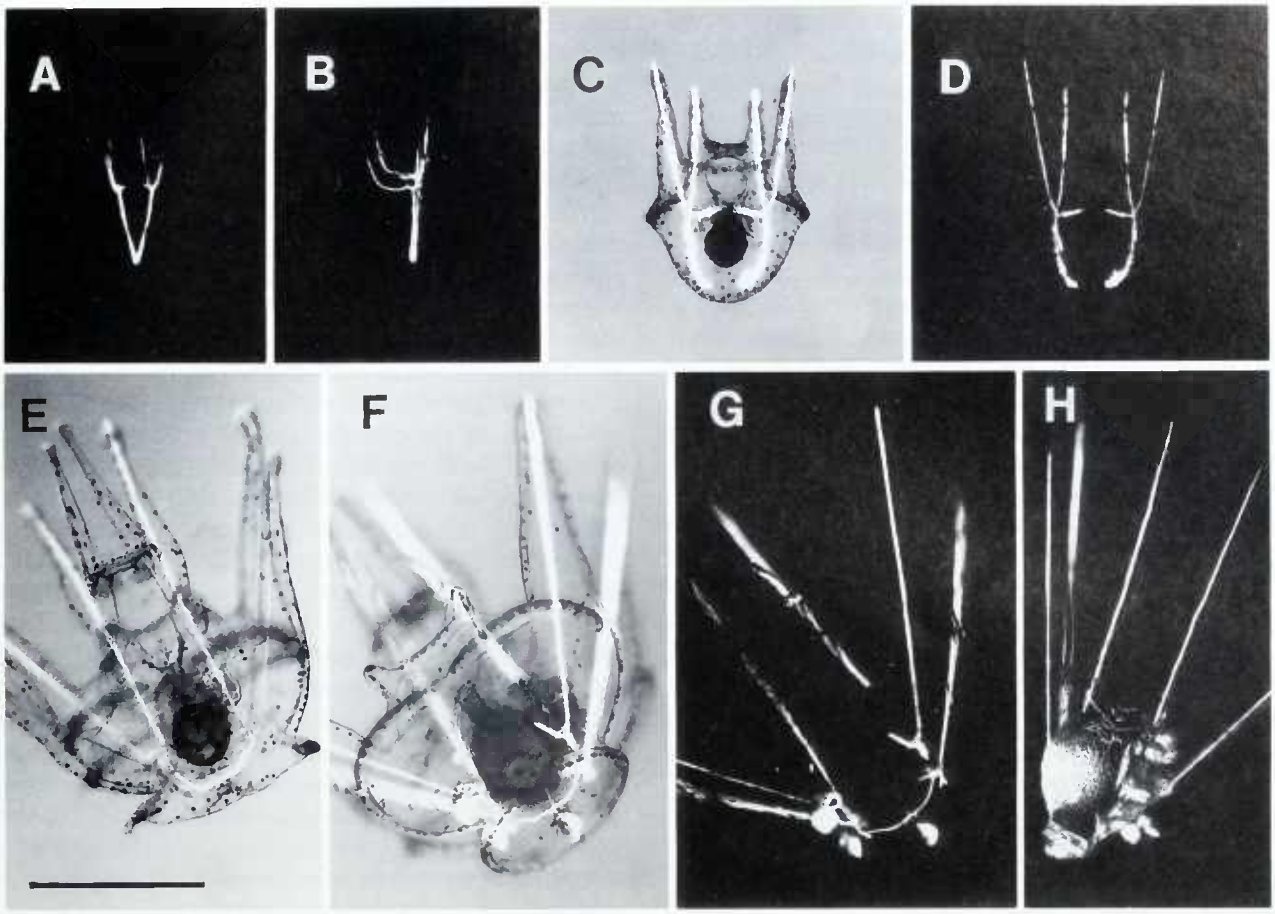

Figure 4. Echinoplutei of *Strongylocentrotus* species: (A) ventral and (B) lateral views of four-armed *S. purpuratus*, (C) and (D) four-armed *S. franciscanus*, (E) eight-armed *S. franciscanus* without pedicellariae, (F) and (G) eight-armed *S. franciscanus* with three pedicellariae, and (H) eight-armed *S. pallidus* with juvenile plates and spines. Calcite skeleton shown with polarizing filters partly crossed in (C, E, F) fully crossed in (A, B, D, G, H). Because of their optical axis the preoral arm rods are not shown. Scale bar is 400 μm for all photos.

rior end upward when in seawater or in low-density mixtures of seawater and isosmotic NaCl and Percoll or in seawater and isosmotic sucrose. However, echinoplutei of *C. atratus*, *E. mathei*, *T. gratilla*, *S. franciscanus*, and *S. pallidus* reoriented with their arms downward as they sank into the denser fluid in the density gradients and approached the depth at which they were neutrally buoyant (Tables I, II). The four-armed plutei of *S. purpuratus* and the plutei of *D. excentricus* and *O. aculeata* did not reorient at neutral buoyancy, nor did auriculariae and bipinnariae. Stability of orientations at neutral buoyancy was demonstrated by changing the positions of larvae with a needle and observing their return to their undisturbed positions.

The reorientation of some plutei in denser fluids was caused by a different distribution of excess mass in dense media. The center of buoyancy was anterior to the center of gravity when the surrounding fluid was seawater, but as these plutei sank into denser fluid, the excess density of the skeleton relative to the fluid decreased, and the excess density of soft tissues and body cavities approached zero and then became negative. The center of buoyancy did not move because the larvae did not change shape, but the center of gravity (defined in terms of excess mass) moved as the density of the surrounding fluid changed. In a denser fluid, therefore, the center of gravity was displaced anterior to the center of buoyancy, and the plutei reversed orientation. These plutei were those with relatively larger body cavities at their posterior ends or relatively lighter skeletons overall (for examples, Fig. 1A, C; 4C–H). These plutei did not reverse orientation simply because they sank into denser fluid than did those that remained anterior end upward; the plutei that remained arms upward at neutral buoyancy (*O. aculeata*, *D. excentricus*, and four-armed *S. purpuratus*) sank into denser fluid than did plutei that reversed their orientation (*S. franciscanus* and *S. pallidus*) (Table II). Thus the differences among plutei in shape and skeletal development and the depths at which their stable orientation was anterior end upward fit our interpretation of the reorientation.

The auriculariae and bipinnariae did not reverse orientation, but that does not imply greater passive stability than the plutei that reversed orientation because the auriculariae and bipinnariae did not sink as far in the density gradient as did these plutei. Nevertheless, the decalcified four-armed plutei of *T. gratilla* reversed their orientation to anterior end downward at neutral buoyancy, whereas the bipinnariae and auriculariae remained oriented anterior end upward. This suggests that a pluteus without a skeleton may have less vertical stability than the bipinnaria and auricularia. This could result from the distribution of epidermal cells, which form a thick columnar layer at ciliated bands and are very thin elsewhere; the ciliated band is placed more anteriorly in plutei than in auriculariae and bipinnariae. Perhaps the skeleton is necessary for the pluteus form because of its contribution to vertical stability, in addition to its role in body support.

Effect of drag on orientation

So far we have ignored the effects of drag on orientation. Drag on swimming larvae could be important but drag cannot account for the following observations on orientation. (1) Echinoplutei of *S. pallidus* and *S. franciscanus* species reoriented with their arms downward as they sank into denser fluid. If drag were responsible for an orientation with arms upward, the larvae would not have reoriented with arms pointing downward as they continued to sink. (2) All four types of echinoderm larvae passively reoriented when turned with a glass needle even when they were at their depth of neutral buoyancy and were no longer sinking. Drag associated with sinking was not necessary to produce stable orientations.

Discussion

The existence and forms of calcite skeletons in feeding echinoderm larvae pose difficult questions. Why should gelatinous planktonic animals have internal mineral skeletons? Why do these larvae have calcite skeletal elements that are unsuited for supporting body parts? How did the complex skeletons of plutei evolve? Answers to these questions depend on functional consequences of larval skeletons. We have examined the importance of the skeleton in passive orientation with two approaches: (1) experimental removal of skeletons from echinoplutei and (2) comparisons among larval forms. We have examined the role of larval skeletons in passive vertical orientation both directly and by the contributions of skeletons to relative density and sinking rates. In this discussion we first review other functional roles that the larval skeleton may have and then explore ways in which a requirement for passive vertical orientation could influence the form and distribution of the calcite skeleton.

Skeletal support for arms. The primary body cavity of echinoderm larvae contains elastic gelatinous material that appears adequate to oppose muscles and to support convoluted body surfaces (Strathmann, 1989). Is a skeleton really needed for the support of larval arms? Experiments indicate that the skeleton does indeed play a role in the development of arms of echinoplutei, but comparisons show that not all arms require a skeleton and that not all calcite skeletal elements support arms. Experiments by Hörstadius (1939), Okazaki (1956), and others showed that an interaction between skeletal rods and the epidermis is necessary for normal development of arms of echinoplutei, though short and rudimentary arms form without skeletal rods. Our removal of skeletons from plutei confirmed these observations. When living plutei with well-developed arms were decalcified, the arms initially became shorter and thicker than the arms of controls (Fig. 2A–C). Within several days the arms completely disappeared in echinoplutei that did not resecrete a skeleton (Fig. 2D). In echinoplutei that resecreted skeletal rods, the arms formed normally, except that length and direction were usually abnormal (Fig. 2E, F).

The skeleton was necessary to maintain the arms of echinoplutei, but skeletal rods are not necessary to support all arms of echinoderm larvae; asteroid larvae lack a calcite larval skeleton, but many late stage asteroid larvae nevertheless develop arms (Mortensen, 1921, 1938). Also, not all skeletal elements support arms; *e.g.*, parts of the body skeleton of plutei and the posterior ossicles of auriculariae. Thus experiments on echinoplutei indicate that the calcite skeletal rods maintain the larval arms; but in some of the other feeding echinoderm larvae skeletal rods are not necessary for the development or support of arms, and the support of arms is not the function of all skeletal elements.

Skeletal defense against predators. Emlet (1983) determined that the fenestrated skeletal rods of arms were stiffer than necessary to support them against forces generated in swimming. Arms of our decalcified live plutei did not discernibly bend or flex during swimming, though admittedly these decalcified arms were very short. Because many arm rods appeared to be much heavier than necessary for support, Emlet (1983) suggested that the skeleton plays a role in defense against predators. The hypothesis is plausible, but direct evidence is lacking. In laboratory experiments, echinoid prism and pluteus stages, which have skeletons, were less vulnerable to predators than were earlier stages that lacked skeletons (Pennington *et al.*, 1986), but this could have been from predator avoidance following development of ciliary arrest and reversal at the late prism stage (Rumrill *et al.,* 1985) rather than from skeletal protection, and the skeleton does not prevent ingestion by crab zoeae (Rumrill and Chia, 1986). A defensive role for the larval skeleton is possible but not yet tested.

Vertical orientation. Runnström (1918) and Pennington and Emlet (1986) suggested that the echinopluteus skeleton weights a larva so that it passively orients with its arms upwards. In such an orientation, forward swimming produces upwards movement that counters sinking. We tested this hypothesis by comparing the vertical orientation of control and decalcified larvae. Vertical orientation and stability of both live and dead four-armed echinoplutei was reduced by removal of the skeleton. Although other effects of temporary exposure to low pH or the shape changes could have affected the vertical orientation of decalcified live larvae, such objections do not apply to the orientation of the dead four-armed echinoplutei without skeletons.

Does the larval skeleton contribute a sufficient part of the total excess mass that it could influence passive orientation? For plutei the answer is "yes," because the small skeleton of a pluteus has a large effect on whole body density and sinking rate. At a salinity of 33‰ and temperature of 25°C the density of seawater is 1.02 g/ml. Therefore, for *T. gratilla,* the estimated excess density of the four-armed echinopluteus without a skeleton was roughly the difference between 1.04 and 1.02 g/ml. The additional contribution of the larval skeleton was the difference between 1.06 and 1.04 g/ml. By this estimate, the skeleton of the four-armed echinopluteus contributed half the excess density of the larva. A similar rough estimate based on the difference in densities of prisms and four-armed plutei of *D. excentricus* (Pennington and Emlet, 1986) suggests that the skeleton of some plutei may contribute as much as 77% of the excess density. If the body volume of four-armed *T. gratilla* did not change with the removal of the skeleton (Fig. 1A, B), then the proportion of the total body mass that was skeleton can be estimated as (1.06–1.04)/1.06, or only about 2% of the body mass. If the density of the skeleton is the same as the density of mineral calcite (2.71 g/ml), then the volume of the skeleton can be estimated as approximately [(1.06–1.04)/2.71] (body volume), or less than 1% of the volume of the larva. These approximate estimates demonstrate that a skeleton of relatively small mass and volume contributed much of the excess mass of the larval body.

The estimated sinking rates of four-armed plutei of *T. gratilla* suggest an even greater contribution of the skeleton to excess density. For slowly sinking objects of this size and the same shape, the sinking rate should be proportional to the excess density (Vogel, 1981). According to the estimated sinking speeds, the plutei with skeletons sank about 5.5 to 8.4 times as fast as the plutei without skeletons. By this estimate, the skeleton provided up to ⅞ of the excess mass of the whole larva. The discrepancy between estimates of sinking rates and estimates of density could result from errors in both estimates. The measured sinking rates were quite variable, and the estimate of density of the larval body could have been biased by the intervals between the densities of test solutions. Although the exact contribution of the skeleton to excess mass is uncertain, the estimated contribution of approximately ½ to ⅞ of excess mass is substantial and indicates that the distribution of skeletal elements is important for the passive orientation of plutei.

Does passive vertical orientation depend on a calcite skeleton for all types of feeding echinoderm larvae? All four types of feeding larvae, even the bipinnariae (with no skeleton), were stable with their anterior ends upward. Also, prior to the deposition of any skeleton, swimming echinoid blastulae and gastrulae swim upward in culture (Lyon, 1906), a presumably passive orientation to gravity. A calcite skeleton can enhance passive vertical orientation, but some degree of vertical orientation and gravitational stability is clearly possible without it.

Does excess density depend on skeletal development for all planktotrophic echinoderm larvae? Or do other features have a greater effect on buoyancy? Comparisons of the varied types of echinoderm larvae showed that larvae with a larger volume of skeleton relative to the volume of soft parts were denser. The plutei were denser than the auriculariae and bipinnariae, and plutei with large skeletal volumes relative to their body volumes were denser than the plutei whose skeletons were relatively small. Also, the passive orientation of plutei with relatively small skeletons was more affected by the distribution of soft parts, as was shown by resting orientation of plutei in solutions denser than their soft parts. Because whole body density increases with the amount of skeleton, there is a functional requirement for the distribution of skeletal parts within the body: if larvae with large skeletons are to maintain passive vertical orientation, the skeleton must have a large posterior component.

Does a skeletal element as small as the posterior ossicle of an auricularia enhance passive orientation? Our observations suggested little contribution of the ossicle of the auricularia of *P. californicus* to excess mass, but we cannot rule out a function in passive orientation. The auriculariae with posterior ossicles had whole body densities similar to those of bipinnariae and of auriculariae without posterior ossicles, and measurements of the ossicles also suggested only a small contribution to excess mass. Most posterior ossicles of *P. californicus* were very flat (Fig. 3D, E, F) and therefore had very small volumes. Nevertheless, the small mass of the ossicle could affect passive orientation because of its extreme posterior position.

Costs of swimming with a skeleton. Larvae with large excess densities must swim to counter sinking. What energetic cost does this impose? To estimate the work necessary to counter sinking, we have combined our estimates for *T. gratilla* with published data for four-armed *D. excentricus.* If a four-armed pluteus has a volume of 2.6×10^{-6} ml (McEdward, 1984) and an excess density of 0.04 g/ml, then the excess mass is 10^{-7} g, and the downward force (F = mg) is 10^{-4} dyne. The product of this force and our estimated sinking velocity of 0.044 cm/s is 4.4×10^{-13} J/s, the rate of work done against gravity. A similar calculation for decalcified plutei with 0.02 g/ml excess density, that sank at 0.0052 cm/s, gives 0.26×10^{-13} J/s, so that the skeleton is responsible for 94% of the work done against gravity. This may be an exaggeration because of the discrepancy between our estimates of sinking rates and excess densities, but even if sinking rates differed by only a factor of 2, as expected from our estimate of excess density, the skeleton would be responsible for 75% of the work done.

However, the total work against gravity appears to be trivially small when compared to total respiratory rate of the larva. An estimate of metabolic capacity for a four-armed pluteus can be converted to a rate of oxygen consumption (McEdward and Strathmann, 1987) and then converted to energy units (4.65×10^{-11} mol O_2/h/larva $\times$ 22.4 l O_2/mol $\times$ 4800 cal/l O_2; conversion factors from Schmidt-Nielsen, 1979) to get 5.8×10^{-9} J/s. The work done against gravity is thereby estimated to be less than 0.01% of the larva's total energy expenditure. The errors in the estimates used in this calculation are undoubtedly large, and the efficiency of the work of swimming is unknown, but the energetic cost of swimming with a skeleton appears to be small. Calculations of work done by swimming copepods (Vlymen, 1970; Alcaraz and Strickler, 1988) and ciliates (Fenchel, 1987) are also a very small part of energy expenditure, as estimated from oxygen consumption. Swimming to counter an increased sinking rate caused by the skeleton apparently does not present an energetic problem for the larvae.

Another consideration is the effect of excess density on rate of ascent. Estimated swimming speeds for feeding larval stages of echinoderms are commonly about 0.3 to 0.5 mm/s (Strathmann, 1971). Konstantinova (1966) estimated 0.3 mm/s for a bipinnaria, but Konstantinova's estimate of 0.1 mm/s for an ophiopluteus appears to be an underestimate. In extensive but unpublished observations, H.-t. Lee measured horizontal swimming speeds of 0.3 to 0.5 mm/s for bipinnariae, up to 0.4 mm/s for auriculariae of *P. californicus,* 0.4 mm/s for two-armed ophioplutei of *O. aculeata,* and 0.4 to 1.9 mm/s for four- to eight-armed echinoplutei of *D. excentricus* and *S. droebachiensis.* The sinking rates of 0.3 and 0.4 mm/s measured for dead four-armed plutei of *T. gratilla* are comparable to these swimming speeds. Plutei would need more propulsion than bipinnariae or auriculariae to achieve the same rate of vertical ascent.

Ultimately, costs of maintaining depth might be independent of excess mass. Echinoderm larvae swim when they feed because the ciliated band produces currents for both swimming and feeding (Strathmann, 1971). If larvae must feed much of the time and if sinking speeds are less than or equal to swimming speeds produced during feeding, then the larvae need only to orient upward to maintain or decrease their depth.

Passive orientation and skeletal form. Because some types of feeding echinoderm larvae orient to gravity without any skeleton, it seems doubtful that the elaborate skeletons of plutei evolved primarily to produce passive vertical orientation. Nevertheless, if vertical orientation is important, its requirements constrain the form of the pluteus skeleton and may explain many features of pluteus morphology. A larger and more elaborate posterior body skeleton is correlated with a larger anterior arm skeleton, as shown by examples in Mortensen (1921, 1938). The thickened posterior body rods in early echinoplutei of the Strongylocentrotidae and Echinidae and the posterior rods of spatangoid plutei may serve as a counterweight for the larval arms.

Similarly, juvenile skeletal elements may be constrained to develop in positions that maintain passive orientation. The asteroid, echinoid, and ophiuroid juvenile rudiments develop in a posterior position.

A role of the skeleton in passive orientation also suggests a functionally advantageous and simple first step in the evolution of larval skeletons. All that is required for passive orientation is a posterior position of the skeletal element; the posterior ossicles in auriculariae may be an example. In contrast, a supporting skeleton must develop as a system of rods favorably placed for supporting muscles or extensions of the ciliated band. Improvements in the skeletal form for support of muscles or projecting arms may have evolved after early formation of calcite ossicles was established.

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Pressure-Temperature Interactions on M_4-Lactate Dehydrogenases From Hydrothermal Vent Fishes: Evidence for Adaptation to Elevated Temperatures by the Zoarcid *Thermarces andersoni*, but not by the Bythitid, *Bythites hollisi*

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Abstract. Lactate dehydrogenases (LDH; M_4 isozyme) were purified from skeletal muscle taken from two fishes endemic to hydrothermal vents, *Thermarces andersoni* (Zoarcidae; 13°N, East Pacific Rise, depth ~ 2600 m) and *Bythites hollisi* (Bythitidae; Galapagos Spreading Center, depth ~ 2500 m), and from the cosmopolitan deep-sea rattail *Coryphaenoides armatus* (Macrouridae; depth of occurrence to ~5000 m). The effects of pressure and temperature on the apparent Michaelis-Menten constant (K_m) of cofactor (NADH) were measured to compare sensitivities to temperature, at *in situ* pressures, of enzymes from hydrothermal vent fishes and from a species adapted to cold, stable deep-sea temperatures. At 5°C, the K_m of NADH of the M_4-LDHs of the three species varied only slightly between measurement pressures of 1 and 340 atmospheres (atm), in agreement with earlier studies of M_4-LDHs of deep-sea fishes. At higher measurement temperatures, marked differences were found among the enzymes. For the M_4-LDHs of *C. armatus* and *B. hollisi*, increases in temperature (10 to 20°C), at *in situ* pressures, sharply increased the K_m of NADH to values higher than those predicted to be physiologically optimal. The M_4-LDH of *T. andersoni* exhibited only minimal perturbation by elevated temperature under *in situ* pressures. The different temperature-pressure responses of these LDHs suggest that enzymes of deep-sea fishes not endemic to hydrothermal vents are not adapted for function at the higher temperatures found at vent sites, and that *T. andersoni* is better adapted than *B. hollisi* for sustained exposure to warm vent waters. The importance of adaptation to warm temperatures in the colonization of vent habitats is discussed.

Introduction

The hydrothermal vent sites at seafloor spreading centers in the Eastern Pacific are, in several ways, unusual deep-sea environments: the food-chain is based on bacterial chemosynthesis rather than photosynthesis; a high degree of endemism characterizes the fauna (Newman, 1985); animal biomass is enormous; and water temperatures are much higher than is typical of the deep sea (~2–3°C) (Hessler and Smithey, 1984; Grassle, 1985). The primary focus of physiological and biochemical research with vent organisms has been on the chemosynthetic processes supporting the food web, and on the adaptations of vent animals to withstand hydrogen sulfide, the primary energy source for chemosynthesis (Grassle, 1985; Somero *et al.*, 1989). Less attention has been paid to the potential importance of temperature as a factor influencing the physiologies of the vent organisms and effecting the distribution of endemic vent species and other deep-sea animals in and near the vent fields.

Temperature typically is a major influence on organismal distribution patterns and physiological function (Hochachka and Somero, 1984; Cossins and Bowler,

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1987), and the steep temperature gradients found at the hydrothermal vents—up to ~380°C over distances of several cm (Fustec *et al.*, 1987)—could present challenging thermal adaptation problems to the vent fauna. Many vent invertebrates encounter temperatures considerably higher than those experienced by deep-sea species living outside the vents. Sessile invertebrates, in particular, live continuously in the warm vent effluents in which temperature can vary between about 2 and 15°C at the Galapagos Spreading Center sites (Hessler and Smithey, 1984; Johnson *et al.*, 1988), and between 2 and at least 20°C at the 13°N site on the East Pacific Rise (EPR) (Fustec *et al.*, 1987). The motile brachyuran crab *Bythograea thermydron* also forages for extended periods in the warm vent waters, and this species appears well adapted for function under conditions of high pressure and elevated temperatures (Arp and Childress, 1981; Mickel and Childress, 1982a, b). Adaptations of hydrothermal vent fishes to high temperature and pressure have not previously been investigated. Although about 20 species of fishes have been described in the general area of the vents (Cohen and Haedrich, 1983), only three fishes, all endemic species, occur within the vent field, and are potentially exposed to waters with elevated temperatures. Two are zoarcids: *Thermarces cerberus* has been identified at both the Galapagos and 21°N site on the EPR; and *T. andersoni* is found at the 13°N EPR site (Rosenblatt and Cohen, 1986). Geistdoerfer (1985), however, regards these two zoarcids as one species.

The hydrothermal vent zoarcids have been observed resting on the basaltic seafloor and, at EPR sites, on the rough surfaces of "smoker" chimneys. EPR sites are characterized by these chimneys which emit hot (up to ~380°C; black smokers) and warmed (~20°C; white smokers) waters (Hekinian *et al.*, 1983). At the EPR sites, cooler water is emitted from fissures in the seafloor. Each vent type—the hot black smokers, the white smokers, and the warm seeps from fissures—has a distinct faunal assemblage associated with it. At all three vent types, *Thermarces* are found in close association with the benthic invertebrates (Fustec *et al.*, 1987). The exact water temperatures encountered by the zoarcids are not known. But, because they have been observed to rest motionless on the bottom among the vestimentiferan tube worms and other invertebrates that live in the warm vent effluents, they may experience warm temperatures for periods long enough to effect thermal equilibration of their bodies with the warm vent waters (Fustec *et al.*, 1987).

The third vent fish described, *Bythites hollisi* (family Bythitidae) (Cohen *et al.*, 1990), has been collected only at the Galapagos Spreading Center, although fishes of similar appearance have been observed from submersibles on the EPR. *B. hollisi* is the only endemic vertebrate common to the Galapagos site (Hessler and Smithey, 1984). Individuals have been observed hovering over warm water vent openings, sometimes with their heads protruding into the cracks from which the warm water is seeping. Given this behavior, *B. hollisi* probably is exposed to water temperatures warmer than ambient deep-sea temperatures. However, the extreme steepness of the thermal gradients above the Galapagos-type warm water vents (up to ~13°C differences over a few cm; see Hessler and Smithey, 1984; Johnson *et al.*, 1988) precludes accurate estimates of the temperatures encountered by *B. hollisi*. Smoker chimneys are absent at the Galapagos site, so there is no potential for *B. hollisi* of this vent habitat to encounter the high temperatures that might confront fishes inhabiting the EPR sites.

A number of fishes typical of the cold deep sea, including rattail fishes (Macrouridae), have been observed swimming near the Galapagos and EPR vent sites (Cohen and Haedrich, 1983). The cosmopolitan rattail *Coryphaenoides armatus* is likely to be found at the depths of the Galapagos Spreading Center and at the 13°N and 21°N EPR sites.

M_4-LDHs have been studied extensively in shallow- and deep-living fishes (Siebenaller, 1987; Siebenaller and Somero, 1978, 1979, 1989), but only at a measurement temperature of 5°C. At this low temperature, the M_4-LDHs of adult fishes occurring at depths greater than 500–1000 m (51–101 atm pressure), differ adaptively from the M_4-LDH homologs of shallow-living, cold-adapted fishes. For example, the effects of pressure on the apparent Michaelis-Menten constant (K_m) of cofactor (NADH) are small or non-existent for the M_4-LDHs of deep-sea species, but very large in the case of the M_4-LDHs of shallow-living fishes. These sharp differences in the effect of pressure on the K_m of cofactor and substrates for LDHs and other enzymes (Siebenaller and Somero, 1989) are hypothesized to play important roles in establishing the depth distribution patterns of marine fishes. Analogously, differences among deep-sea species in the effects of temperature on their enzymes under *in situ* pressures might play a role in determining horizontal distribution patterns related to temperature gradients near hydrothermal vent sites.

To determine whether differences in temperature adaptation exist between the biochemistries of endemic vent fishes and deep-sea fishes from cold, thermally stable waters, we studied the skeletal muscle isozymes (M_4 = A_4) of lactate dehydrogenase (LDH; EC 1.1.1.27); the kinetic and structural properties of this enzyme strongly reflect the temperatures and pressures to which an organism is adapted (Yancey and Somero, 1978; Siebenaller and Somero, 1989). M_4-LDHs from *T. andersoni*, *B. hol-*

lisi, and *C. armatus* were purified and studied kinetically over a range of pressures and temperatures to determine how temperatures typical of warm water vents affect the response of M_4-LDHs to *in situ* pressures.

Materials and Methods

Collection and preservation of specimens

The specimen of *B. hollisi* (initial description by Cohen *et al.*, 1990) was captured by net from the DSV *Alvin* at the Galapagos Spreading Center during the Galapagos-1988 expedition. The specimen was returned to the surface in an insulated container and immediately dissected. Muscle samples were frozen immediately in liquid nitrogen, and returned to the Scripps Institution of Oceanography (SIO) for analysis.

The specimen of *T. andersoni* was captured in a baited trap at the 13°N EPR site during the autumn 1987 French-US Hydronaut expedition. Recovery was achieved using the French submersible DSV *Nautile*. The specimen was frozen immediately upon return to the ship, returned to SIO, and stored at −80°C until analyzed.

C. armatus was collected by otter trawl in Monterey Canyon at a depth of ~3000 m. White muscle was dissected from the fish, wrapped in aluminum foil, and frozen immediately on dry ice. Tissues were returned to SIO and stored at −80° until analyzed.

Enzyme purification and determinations of K_m of NADH

The M_4 isozyme of LDH was purified with an oxamate affinity column, as described by Yancey and Somero (1978). Native starch and polyacrylamide gels stained for LDH activity revealed a single band of activity, the M_4-LDH. SDS-polyacrylamide gels stained with Coomossie blue showed a single protein band corresponding in M_r to LDH.

The K_m of NADH was determined using an 80 m*M* imidazole/Cl buffer (pH 7.0 at 20°C). This buffer was chosen, rather than the Tris/Cl buffer used in earlier studies of the effects of pressure on LDH (*cf.* Siebenaller and Somero, 1978, 1979), because the pK of imidazole varies with temperature in parallel with the intracellular pH (pH_i) of fish muscle (Reeves, 1977). The pH values of imidazole/Cl buffers, like those of Tris/Cl buffers, are virtually unaffected by pressures in the range used in these studies (Kauzmann *et al.*, 1962). Except for the differences in assay medium (buffer species and KCl concentration; *cf.* Siebenaller and Somero, 1978), the high pressure assays were made following the protocol of Siebenaller and Somero (1978). Seven to nine concentrations of NADH spanning the value of K_m were used to determine each K_m value. The K_m values were computed according to the weighted linear regression method of Wilkinson (1961) (Wilman4 software; Brooks and Suelter, 1986). Standard deviations of the K_m values did not exceed 12% of the K_m values (Fig. 1).

Results

The effects of temperature on the pressure sensitivities of the K_m of NADH for the M_4-LDHs of the three species are illustrated in Figure 1. At 5°C, the kinetics of these enzymes resembled those of the high pressure-adapted M_4-LDHs of other deep-sea fishes (see Siebenaller, 1987; Siebenaller and Somero, 1989). Increased pressure caused at most a slight increase in the K_m of NADH, and this increase occurred over the first 68 atm rise in measurement pressure. Pressures above 68 atm caused no further increase in K_m.

At temperatures above 5°C, the M_4-LDH of *T. andersoni* differed from the homologs of the other two deep-sea species (Figs. 1, 2). At *in situ* pressures (~250 atm; dashed vertical line in Fig. 1), the M_4-LDH of *T. andersoni* exhibited no increase in K_m of NADH between 5 and 10°C, and only a slight increase between 10 and 20°C. The M_4-LDHs of *C. armatus* and *B. hollisi* exhibited an approximate doubling of the K_m of NADH as the temperature increased to 15 or 20°C.

Discussion

The K_m of substrate or cofactor for a given type of enzyme is strongly conserved among species at their physiological temperatures (Yancey and Siebenaller, 1987; Yancey and Somero, 1978) and pressures (Siebenaller, 1984, 1987; Siebenaller and Somero, 1978, 1989). The K_m of NADH for M_4-LDH varies at most by about 10 μM, both among species at their physiological temperatures and pressures, and across a single species' normal range of body temperatures and pressures. At temperatures or pressures above the normal physiological range, the K_m of NADH typically exhibits a large temperature- or pressure-related increase, and reaches values that no longer lie within the conserved range that is viewed as physiologically optimal. Similar trends have been seen for several enzymes, which emphasizes that enzymatic kinetic properties must be maintained within narrow ranges that are optimal for catalysis and regulation (reviewed by Hochachka and Somero, 1984; Siebenaller and Somero, 1989). Conservation of K_m and other kinetic parameters may only be observed when comparative studies of enzyme homologs are all performed in the same *in vitro* milieu; differences in ionic strength, for example, can affect the absolute values of K_m (*cf.* Siebe-

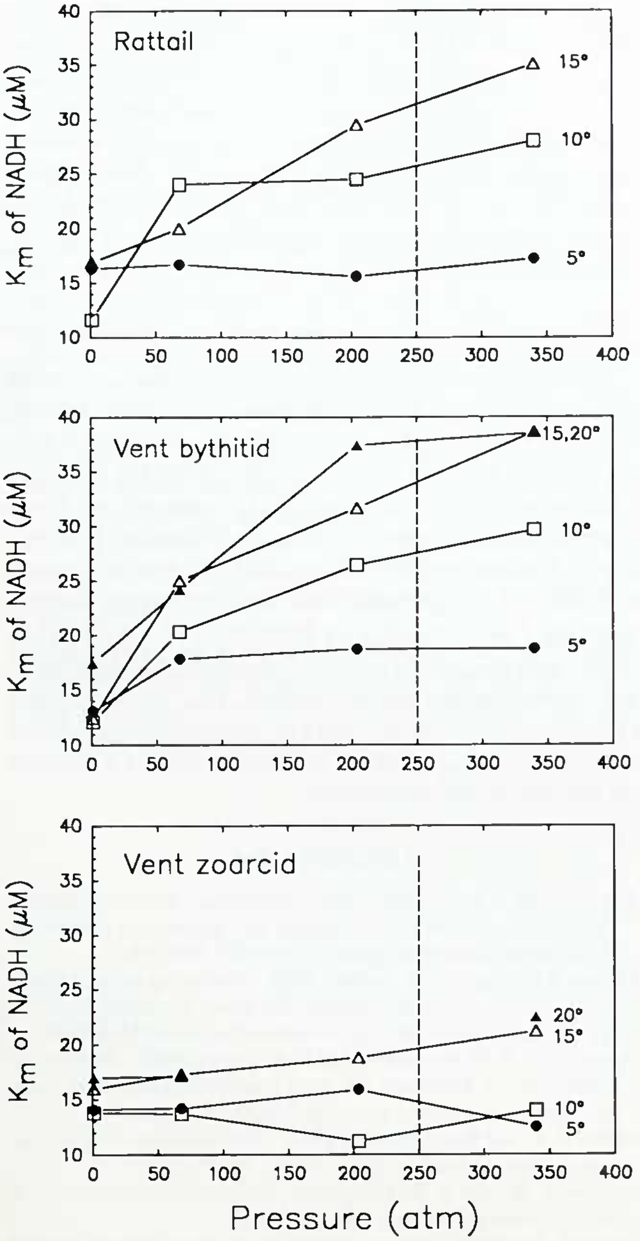

Figure 1. The effects of measurement temperature and pressure on the apparent Michaelis-Menten constant (K_m) of NADH for M_4-LDHs of the cosmopolitan deep-sea rattail fish *Coryphaenoides armatus*, the hydrothermal vent bythitid *Bythites hollisi*, and the hydrothermal vent zoarcid *Thermarces andersoni*. The dashed vertical line indicates the approximate habitat pressure at the two vent sites.

naller and Somero, 1978, with Yancey and Siebenaller, 1987).

For the M_4-LDHs of *C. armatus* and *B. hollisi*, temperatures of 10 to 20°C increased the K_m of NADH by ~15–20 μM at *in situ* pressures (Fig. 2). In contrast, the K_m of NADH for the M_4-LDH of *T. andersoni* increased by only approximately 8 μM as temperature increased from 5 to 20°C. Therefore, temperatures characteristic of warm water vents perturbed the K_m of NADH of the M_4-LDHs of *C. armatus* and *B. hollisi* sufficiently to increase their values beyond the physiologically conserved range noted for other species. The M_4-LDH of *T. andersoni* retained its K_m of NADH within the physiologically conserved range across the span of measurement temperatures at *in situ* pressure.

The different responses of the M_4-LDHs of these three species to changes in temperature at *in situ* pressure lead us to propose two hypotheses concerning the relationship between species distribution patterns and temperature and pressure influences on enzymatic function. First, we propose that the M_4-LDHs of cold-adapted deep-sea fishes are not pre-adapted for function at the elevated temperatures found at the warm water vents. Thermal perturbation of the kinetic properties of enzymes under pressure may restrict the endemic fauna of the cold deep sea from exploiting hydrothermal vent habitats. Thus, as much as interspecific differences in the pressure sensitivities of enzymes may be important in establishing species' vertical distribution patterns in the marine water column (Siebenaller and Somero, 1989), interspecific differences in the responses of enzymes to elevated temperatures, at deep-sea pressures, may be instrumental in establishing horizontal distribution patterns in temperature gradients near the deep-sea hydrothermal vents. This conjecture is not meant to imply that temperature is the only factor restricting typical deep-sea animals from the vent environment. Mechanisms for overcoming the toxic effects of hydrogen sulfide also ap-

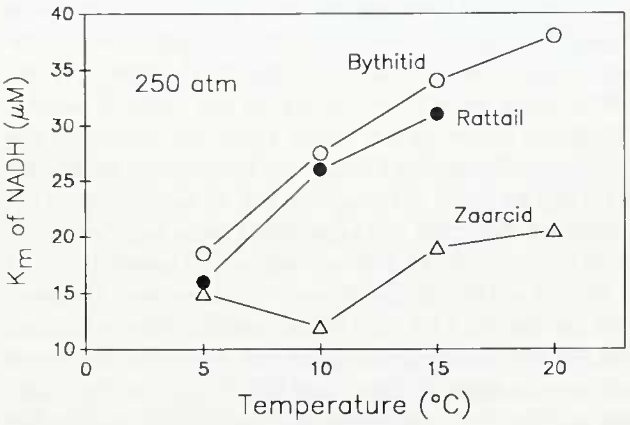

Figure 2. The effect of measurement temperature, at the approximate habitat pressure of the two hydrothermal vent sites (~250 atm), on the K_m of NADH for the M_4-LDHs of the three species shown in Figure 1. K_m values at 250 atm were estimated by the intersection of the vertical dashed line (corresponding to 250 atm pressure) with the lines connecting the K_m values at each temperature (see Fig. 1).

pear to be important components of adaptation to the vent environment (Somero *et al.*, 1989).

Second, we hypothesize that, among endemic vent species, there may be substantial differences in tolerance of high temperature and, therefore, in the microhabitats they experience. The interacting effects of elevated temperature and pressure on its M_4-LDH suggest that *B. hollisi* from the Galapagos Spreading Center is not adapted for continuous existence in the warmest waters found at this site. In contrast, by our enzymatic criterion, *T. andersoni* appears well adapted to body temperatures as high as 20°C.

Because the exact temperatures experienced by endemic vent fishes, and the times over which they remain in warm waters, are not known with accuracy, links between enzymatic properties and environmental distributions remain speculative. However, the contrasting thermal properties of their environments suggest that the two vent fishes used in these studies have different thermal experiences. At the Galapagos Spreading Center site, where *B. hollisi* is the most abundant endemic vertebrate, smoker chimneys are absent, and the highest temperature recorded in the warm water vents was ~15°C (Johnson *et al.*, 1988). Although *B. hollisi* is commonly found hovering over the vent openings, and may even enter the sites of venting (Robert R. Hessler, Scripps Institution of Oceanography, pers. comm.), the extremely steep thermal gradients characteristic of the vents make precise estimates of the fish's body temperature impossible. *B. hollisi,* unlike *T. andersoni,* appears to spend most of its time swimming and, therefore, may select water temperatures that are lower than those encountered by the demersal zoarcid, which commonly rests among sessile invertebrates living directly in the warm vent effluent. At the 13°N EPR site, where *T. andersoni* is the most abundant endemic vertebrate, the temperatures of the warm water vents reach at least 20°C (Fustec *et al.*, 1987). Zoarcids are also found on the walls of smoker chimneys, where waters much hotter than those at the Galapagos Spreading Center are emitted. Zoarcids are observed to swim very rapidly out of hot smoker-vent waters, so they may not experience these high temperatures for more than a few seconds per encounter.

Recent studies of the effects of pressure and temperature on the K_ms of NADH of malate dehydrogenases (MDHs) of invertebrates from the hydrothermal vents and several other shallow- and deep-water marine habitats support the hypothesis that adaptation to elevated temperatures is important for vent species exposed to warm vent effluents for extended periods (Dahlhoff, 1989; Dahlhoff and Somero, in prep.). Although all of the MDHs from deep-sea invertebrates were found to be pressure insensitive at 5°C, only the warm-adapted hydrothermal vent species exhibited the pattern of stability of the K_m of NADH under high pressure and elevated temperature shown here for the M_4-LDH of *T. andersoni.* We propose, then, that the hydrothermal vent animals, which attain thermal equilibrium with the warm vent waters, are characterized by pervasive biochemical adaptations to elevated temperatures, and these adaptations are prerequisite to an exploitation of the warm microhabitats in the vent field.

Acknowledgments

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Extraction of a Vanadium-Binding Substance (Vanadobin) from the Blood Cells of Several Ascidian Species

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Abstract. A combination of techniques, including chromatography on Sephadex G-15 and SE-cellulose columns and neutron activation analysis for vanadium determination, was used to extract (at low pH) a vanadium-binding substance (vanadobin) from the blood cells of the ascidian species: *Ascidia ahodori* OKA, *A. gemmata* SLUITER, *A. zara* OKA, *Corella japonica* HERDMAN, and *Ciona intestinalis* (LINNE). In general, ascidians can be classified into two different categories based on vanadium content: species of the family Ascidiidae contain high levels of vanadium, whereas those in the Cionidae and Corellidae do not always have such high amounts. Because *Ciona intestinalis* and *Corella japonica* do have vanadobin in their blood cells, vanadobin may well be a universal complex in ascidians, having the role of accumulating vanadium in blood cells and maintaining its concentration. The blood cells of *A. gemmata* contained the highest amount of vanadium. Vanadobin extracted from these cells exhibits absorption spectra, not only in the ultraviolet region, but also in the visible region; such spectra correspond to those observed in vanadium complexes in oxidation states of +3 and +4.

Introduction

The unusual ability of ascidian blood cells to accumulate vanadium in excess of one million times its level in seawater has attracted the interest of investigators from various fields of study. In particular, the chemical form of the vanadium complex present in ascidian blood cells has long been a subject of discussion. Sometime after Henze's first discovery of vanadium in ascidian blood cells (Henze, 1911), the element was believed to occur as part of a sulfated nitrogenous compound known as haemovanadin (Califano and Boeri, 1950; Webb, 1956; Bielig *et al.*, 1966). Kustin's group claimed that haemovanadin is an artificial product generated by air oxidation (Kustin *et al.*, 1976; Macara *et al.*, 1979a, b).

The vanadium ion dissolved in seawater seems to exist as the vanadate(V) anion in the +5 oxidation state (McLeod *et al.*, 1975), but this is still experimentally unresolved (Biggs and Swinehart, 1976). The vanadium ion contained in ascidian blood cells is, however, reduced to +4 or +3 oxidation states, *i.e.*, vanadyl cations (*cf.* Michibata and Sakurai, 1990). It has therefore been assumed that agents causing the reduction of vanadate ion to vanadyl ion must occur within ascidian blood cells and there bind with the vanadium ion. Kustin's group isolated a tunichrome from ascidian blood cells: it was proposed as being involved in the accumulation of vanadium and in its reduction from seawater (Macara *et al.*, 1979a, b; Bruening *et al.*, 1985). However, there is still no evidence that this tunichrome fulfills those functions in vanadium-containing blood cells (vanadocytes). Furthermore, the fluorescence due to the tunichrome is certainly not detected in the signet ring cells that have been identified as the vanadocytes (Michibata *et al.*, 1988, 1990a).

While extracting tunichrome, Gilbert *et al.* (1977) and Macara *et al.* (1979a) found a vanadium-containing band upon Sephadex column chromatography, but they continued to focus mainly on characterizing the tuni-

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chrome. More recently, in contrast, while characterizing a vanadium-binding substance extracted from the blood cells of *Ascidia sydneiensis samea* OKA under acidic conditions, we showed that this substance, named vanadobin, could maintain the vanadium ion in the vanadyl form (VO(IV)), had an apparent affinity for vanadium ion, and contained a reducing sugar (Michibata *et al.*, 1986a).

The present investigation was designed to determine whether vanadobin could be extracted from the blood cells of other ascidians that contain significant amounts of vanadium and, thus, to ascertain whether vanadobin is a universal characteristic of blood cells in vanadium-containing ascidians. In fact, vanadobin was extracted from the blood cells of all ascidian species examined. Furthermore, the vanadobin extracted from *A. gemmata* shows an absorbance in the visible range resembling that of a vanadium compound.

Materials and Methods

Ascidia ahodori OKA, *A. zara* OKA, and *Ciona intestinalis* (LINNE) were collected from the Ushimado Marine Biological Station of Okayama University in Ushimado, Okayama Prefecture, Japan. *A. gemmata* SLUITER was obtained from the Asamushi Marine Biological Station of Tohoku University in Asamushi, Aomori Prefecture. *Corella japonica* HERDMAN was gathered in Yamada Bay near the Ootsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo in Ootsuchi, Iwate Prefecture.

Blood from each species was collected by cardiac puncture under an anaerobic atmosphere of nitrogen gas to preclude air-oxidation; subsequent techniques were also carried out under the same conditions. The blood cells were separated from the blood plasma by centrifugation at 3000 × *g* for 20 min at 4°C, and were pooled at −80°C before use. A 10 m*M* glycine-HCl buffer solution at pH 2.3 was added to the cell pellet, and the suspension was then ground in a glass-Teflon homogenizer at 4°C. For *A. gemmata* and *Corella japonica*, glycine-HCl buffer was substituted with a 50 m*M* HCl solution throughout the experimental process.

The homogenate was loaded onto a column of Sephadex G-15 (Pharmacia Fine Chemicals). The column size and the chromatographic parameters used with each species is described in the appropriate figure legend. The column was equilibrated with the glycine-HCl buffer solution, and the elutant was collected in 3 ml or 5 ml fractions with monitoring for UV absorbance.

The amounts of vanadium in each blood cell pellet and in the fractions were measured by neutron activation analysis or atomic absorption spectrometry. For neutron activation analysis, the elutant of each fraction was repacked in a polyethylene capsule and irradiated with thermal neutrons having a flux of 5×10^{11} n/cm^2 $\times$ s^{-1} for 2 min in a TRIGA MARK II nuclear reactor at the Institute for Atomic Energy, Rikkyo University, Yokosuka, Japan. The radioactivity of ^{52}V produced in the irradiated sample was measured with a 50-cm^3 Ge(Li) γ-ray spectrometer (Canberra Inc.) 2 min after the irradiation. Using a photon energy for ^{52}V of 1432KeV, the amount of vanadium in the sample was determined by comparison with that of a standard, as described previously (Michibata *et al.*, 1986b). Flameless atomic absorption spectrometry was applied to the samples that contained relatively higher amounts of vanadium. For these measurements we used a Hitachi GA-2 with a graphite furnace, and an absorption line of 3183.9 A was used for vanadium determination.

The absorbance of fractions observed to contain vanadium was measured with a spectrophotometer (Hitachi U-3210); the fractions were then pooled and loaded onto a column of SE-52 (Serva Feinbiochimica) for ion-exchange chromatography (Whatman W. & R.) and, thus, for further purification. Non-absorbed substances could be washed off the column with a sufficient volume of 10 m*M* glycine-HCl buffer, and the purer vanadobin could then be eluted with a linear gradient of KCl, from 0 to 0.5 *M* in the buffer solution. The vanadium content in each fraction obtained was also measured by the methods described above, and absorbance was monitored with a spectrophotometer.

We examined whether a simulated absorption spectrum of vanadobin from *A. gemmata* could be reproduced using spectra of inorganic vanadium complexes. Vanadium(III) sulfate ($V_2(SO_4)_3$) was dissolved in 30 m*M* H_2SO_4, in a concentration of 40 m*M* at pH 1, under an anaerobic atmosphere of argon gas, and vanadium(IV) oxide sulfate ($VOSO_4$) was dissolved in distilled water (10 m*M*, at pH 2.0 to 3.5 adjusted with 1 *M* HCl). Each absorbance was measured with a spectrophotometer, and each molar extinction coefficient was calculated. Based on these data, a spectrum closely resembling that of vanadobin was calculated.

Results

Ascidia gemmata *SLUITER*

This species contained a higher level of vanadium in its blood cells than any other species examined (8.75 μg/mg wet weight) (Table I). Figure 1 shows the elution profile that resulted when a homogenate of the blood cell pellet (1.64 g wet weight) of *A. gemmata* was loaded onto Sephadex G-15 and eluted. A large peak in fractions 8 through 22 was observed. As shown in Figure 2, the spec-

Table I

Extraction process of vanadobin from the blood cells of several ascidian species through chromatography

	Pellet of blood cells		Sephadex G-15		SE-cellulose	
	Wet weight (mg)	V content (μg)	V content (μg)	Abs. max. (nm)	V content (μg)	Abs. max. (nm)
Ascidia gemmata	1640.0	14,350.0 (100.0%)	13,043.5 (91.0%)	245, 410, 756	8347.8 (64.0%)	238, 756
Ascidia ahodori	49.7	101.4 (100.0%)	62.5 (61.6%)	254	25.9 (25.5%)	232
Ascidia zara	40.1	140.5 (100.0%)	30.6 (21.3%)	236		
Corella japonica	13,900.0	269.0 (100.0%)	236.0 (87.7%)	262	222.8 (82.8%)	260
Ciona intestinalis	86.3	22.5 (100.0%)	6.9 (30.7%)	246		

V content: vanadium content, Abs. max.: absorption maximum. The recovered vanadium through the chromatography is expressed as percent of the initial amount in the pellet of blood cells in parentheses.

trum of the peak fraction (fraction 13) had a clear ultraviolet peak at 245 nm, and absorption peaks at 410 nm and 756 nm in the visible range with a shoulder at 620 nm. The elution profile of vanadium coincided with the peak of absorbance. The total vanadium content detected in fractions 8 through 20 was 13043.5 μg.

The fractions from Sephadex G-15 column chromatography that contained vanadium were loaded onto a column of SE-52, providing the profile shown in Figure 3. By eluting with a gradient of KCl, several small peaks containing no vanadium were washed off the column, and then a sharp peak containing vanadium was obtained in fractions 199 through 216. The amount of vanadium recovered was 8347.8 μg. The peak fraction (fraction 205) exhibited absorption maxima at 236 nm and 756 nm, as shown in Figure 4. *A. gemmata* was the only species in which vanadobin exhibited absorption peaks in the visible range.

Absorption spectra of vanadium(III) sulfate and vanadium(IV) oxide sulfate

Absorption spectra of vanadium(III) sulfate and vanadium(IV) oxide sulfate are shown in Figures 5a and b, respectively. The former had absorption peaks at 400 nm and 610 nm, and the latter a peak at 760 nm with a shoulder at 625 nm. When vanadium(III) sulfate ($V_2(SO_4)_3$)

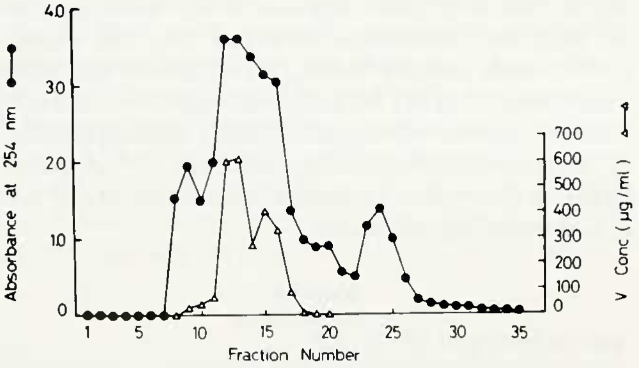

Figure 1. Elution profile of the blood cell homogenate of *Ascidia gemmata* on Sephadex G-15 column chromatography. Pelleted blood cells (1.64 g) were homogenized in about 50 m*M* HCl buffer solution, which was adjusted at pH 2.3. The homogenate (14 ml) was loaded onto a column (1.5 cm $\phi \times 48$ cm) and eluted with the same solution in 5-ml fractions. The total bed volume (Vt) and the void volume (Vo) of the column were 85 ml and 39 ml, respectively. The elution volume (Ve) of vanadobin was 65 ml.

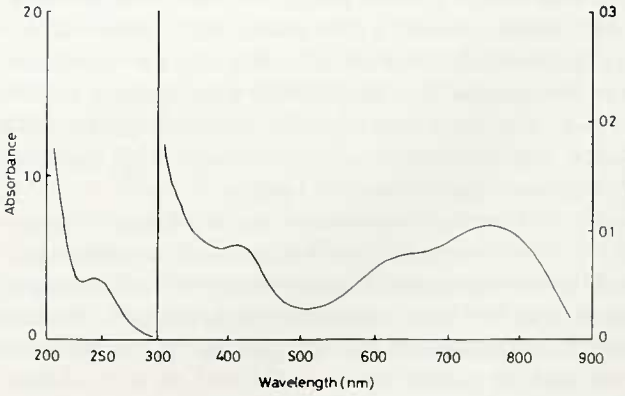

Figure 2. Absorption spectrum of vanadobin eluted from Sephadex G-15 column chromatography. The spectrum was recorded for the peak fraction 12 shown in Figure 1. Absorbance at 245 nm, 410 nm, and 756 nm with a shoulder 620 nm was observed.

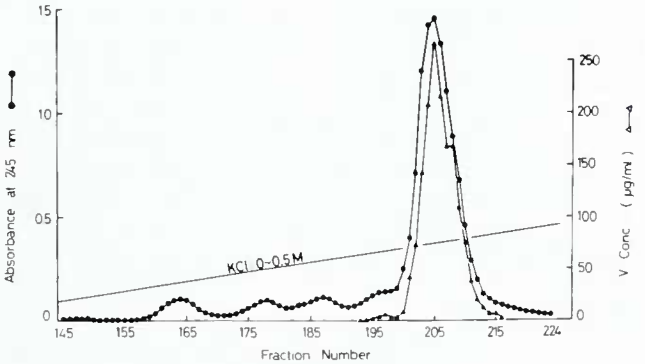

Figure 3. Elution profile of the vanadium-containing substance (vanadobin) of *Ascidia gemmata* on SE-cellulose column chromatography. Vanadium-containing fractions (45 ml) obtained after passing through Sephadex G-15 were loaded onto a column (3.7 cm ϕ × 25 cm). After non-absorbed substances were washed off the column by eluting with 50 m*M* HCl solution at pH 2.3 in 5 ml fractions, the vanadobin was obtained by elution with a linear gradient of KCl at a concentration of 0.25 *M*.

and vanadium(IV) oxide sulfate ($VOSO_4$) were mixed together at concentrations of 4.8 m*M* and 5.9 m*M*, respectively, an absorption spectrum closely resembling that of vanadobin after elution through Sephadex G-15 (Fig. 2) was obtained, as shown in Figure 5c. This simulation clearly suggested that the vanadobin of *A. gemmata*, which was eluted from Sephadex G-15, contained vanadium in the +3 and +4 oxidation states in the ratio of 45:55, whereas for the purer vanadobin eluting from SE-cellulose, the absorption peak at 410 nm disappeared, and a peak at 756 nm with a shoulder at 620 nm was seen (Fig. 4). These findings indicate that, although vanadobin originally contains both vanadium forms in the +3 and +4 oxidation states, vanadium in the +3 oxidation state becomes oxidized to the +4 oxidation state during the purification.

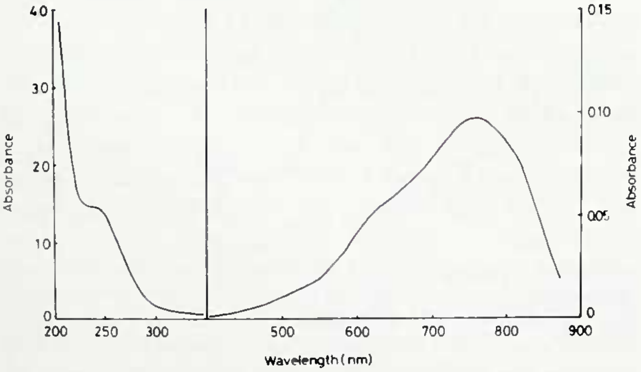

Figure 4. Absorption spectrum of vanadobin eluted from SE-cellulose column chromatography. The spectrum was recorded for the peak fraction 205 shown in Figure 3. Absorbance at 238 nm and 756 nm was observed.

Ascidia ahodori *OKA*

When a homogenate containing 49.7 mg wet weight of the cell pellet was loaded on the Sephadex G-15 col-

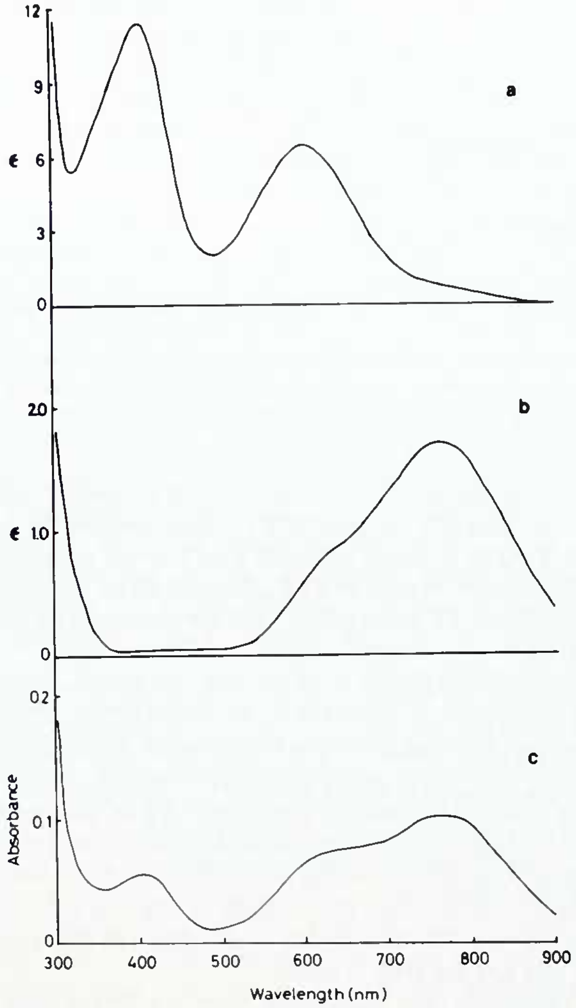

Figure 5. Absorption spectra of vanadium complexes. a. Vanadium(III) sulfate ($V_2(SO_4)_3$) was dissolved in 30 m*M* H_2SO_4 in a concentration of 40 m*M* at pH 1 under an anaerobic atmosphere of argon gas. Absorbance is expressed as (molar extinction coefficient). b. Vanadium(IV) oxide sulfate ($VOSO_4$) was dissolved in distilled water in a concentration of 10 m*M* at pH 2.0 to 3.5. Absorbance was expressed as (molar extinction coefficient). c. Simulated spectrum, closely resembling that of the vanadobin shown in Figure 2, was obtained when vanadium(III) sulfate ($V_2(SO_4)_3$) and vanadium(IV) oxide sulfate ($VOSO_4$) were mixed together at respective concentrations of 4.8 m*M* and 5.9 m*M*. This simulation suggested clearly that the chemical forms of vanadium in the +3 and +4 oxidation states were approximately in the ratio 5:6 in the vanadobin of *A. gemmata* eluted from Sephadex G-15 column (Fig. 2).

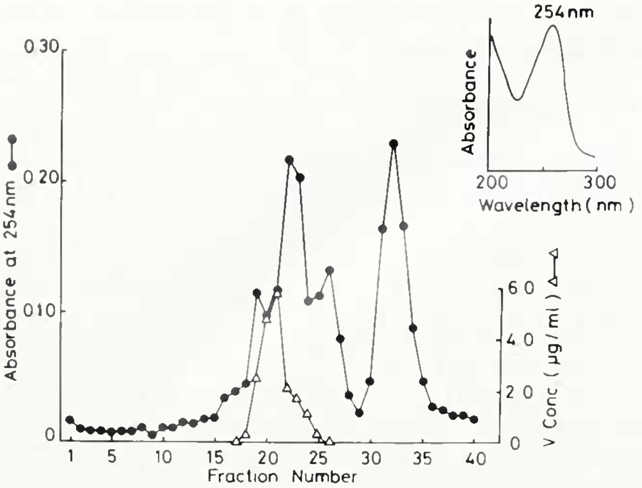

Figure 6. Elution profile of the blood cell homogenate of *Ascidia ahodori* on Sephadex G-15 column chromatography. Pelleted blood cells (49.7 mg) were homogenized in 10 mM glycine-HCl buffer solution at pH 2.3. The homogenate (1.2 ml) was loaded onto a column (1.5 cm $\phi \times$ 48 cm) and eluted with the same buffer solution in 3 ml fractions. Vt and Vo of the column were 85 ml and 39 ml, respectively. Ve of vanadobin was 63 ml. The inset shows the UV spectrum of the peak fraction (fraction 21). Absorbance at 254 nm was observed.

umn, the elution profile obtained was somewhat more complicated (Fig. 6). A scan of UV wavelengths for fraction 21 (Fig. 6, inset) indicates that 254 nm is the peak of absorption. A total of 62.5 μg of vanadium was eluted in fractions 17 through 26, and its peak was in fraction 21.

As shown in Figure 7, when these vanadium-containing fractions were loaded onto a column of SE-52, non-absorbed substances were first washed off the column by eluting with the glycine-HCl buffer solution, and a big peak with no vanadium was eluted with a linear gradient of KCl dissolved in buffer solution. The vanadobin was obtained thereafter in fractions 54 through 58 with a peak in fraction 56. The vanadium content recovered was 25.9 μg. The absorption peak, observed in fraction 56, was 232 nm (Fig. 7, inset).

Vanadobin was extractable from the blood cells of three other ascidian species, *A. zara* OKA, *Corella japonica* HERDMAN, and *Ciona intestinalis* (LINNE); the procedures and results were similar to those described above (data not shown). The process of vanadobin extraction by chromatography is summarized in Table I. The highest vanadium content of 8750 ng/mg wet weight (14350 ng/1640 mg wet weight) in the blood cells was observed in *A. gemmata*. The next highest value was 2040 ng/mg wet weight detected in the blood cell pellet from *A. ahodori*. The blood cells of *Ciona intestinalis* had a much smaller content of 261 ng/mg wet weight of vanadium. *Corella japonica*, a vanadium-poor species, contained the smallest amount in its blood cells 19 ng/mg wet weight). Recovery rates of vanadium ion in fractions eluted through Sephadex G-15 were in the range of about 21–90%. The rates through SE-cellulose were in the range of about 26–83% of each initial amount. Peak wavelengths of UV absorbance in the peak fraction of Sephadex G-15 were observed between 246 nm and 262 nm, and the values observed in the peak fraction of SE-cellulose were shifted to shorter wavelengths, specifically to about 232 nm.

Discussion

Previous observations notwithstanding (Michibata *et al.*, 1986a), our present experiments show that vanadobin extracted from ascidian blood cells does, in fact, exhibit clear absorption peaks in the visible range when its contained vanadium in vanadobin is at a significantly high concentration. In the case of *A. gemmata*, the concentrations of vanadium in the peak fractions eluted from Sephadex G-15 and SE-cellulose columns were estimated to be 12.3 mM and 5.3 mM, respectively. The successful detection of this visible absorption is due to the high level of vanadium in the blood cells of *A. gemmata* than any other ascidian, and to the extremely large volume of sample loaded onto the column (*cf.* Table I).

Because the absorption spectrum of vanadobin from *A. gemmata* resembled those of vanadium compounds, an attempt was made to obtain a simulated spectrum.

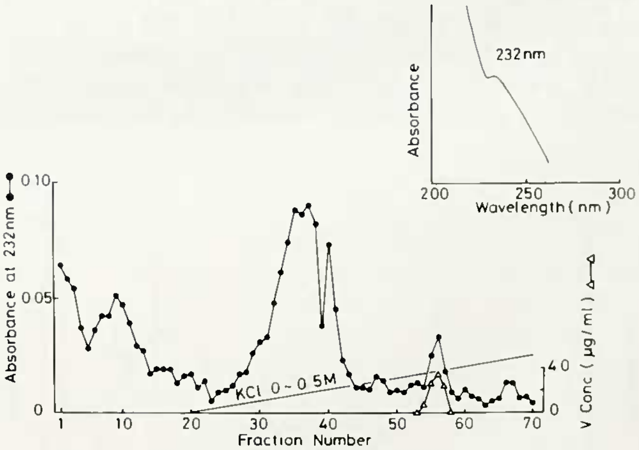

Figure 7. Elution profile of the vanadium-containing substance (vanadobin) of *Ascidia ahodori* on SE-cellulose column chromatography. Vanadium-containing fractions (15 ml) obtained after passing through Sephadex G-15 were loaded onto a column (1.2 cm $\phi \times$ 12 cm). After non-absorbed substances were washed off the column by eluting with 10 mM glycine-HCl buffer solution at pH 2.3 in 3 ml fractions, vanadobin was obtained by elution with a linear gradient of KCl (0.1 M). The inset shows the UV spectrum of the peak fraction (fraction 56). Absorbance at 232 nm was observed.

Indeed, the spectrum of vanadobin eluted from Sephadex G-15 (Fig. 2) can be reproduced when vanadium compounds in the +3 and +4 oxidation states are mixed together in a ratio of 45:55 (Fig. 5c). We therefore conclude that vanadobin contains vanadium in the +3 and +4 oxidation states in this ratio. On the other hand, the peak at 410 nm disappears from vanadobin after elution through SE-cellulose (Fig. 4) because vanadium(III) is oxidized to vanadium(IV) during the experimental process.

The present report is the first to document an obvious absorption peak in the visible range for the native vanadium complex, vanadobin. Previously, an absorbance peak in the visible range at 430 nm had been reported for a blood cell lysate of *Ascidia obliqua* (Boeri and Ehrenberg, 1954); the 430-nm peak was attributed to the hydrolyzed ion $V(OH)_2V^{4+}$ (or VOV^{4+}). Brand *et al.* (1989) also found visible-range absorption of vanadium complexes from ascidian blood cells after addition of exogenous ligands, 2,2′-bypyridine, 1,10-phenanthroline, and acetylacetone.

Recent analysis of ascidian blood cells by NMR (nuclear magnetic resonance), EXAFS (extended X-ray absorption fine structure), and SQUID (superconducting quantum interference device) has suggested that the vanadium is present predominantly in the +3 oxidation state, and that the +4 state accounts for less than 10% (Carlson, 1975; Tullius *et al.*, 1980; Lee *et al.*, 1988). The present results confirm that data obtained from the visible spectrum are also available for further determination of the valency of vanadium in cases where a high concentration of the metal is present in ascidian blood cells.

The possibility has existed that vanadobin is an artificial complex produced during the experimental process. That is to say, although the signet ring cell, among several types of ascidian blood cells, is clearly the vanadium-containing blood cell (the vanadocyte) (Michibata *et al.*, 1987), the vanadium contained in the vanadocyte could well be mixed with a substance that was apt to bind with the metal contained in another cell type. Consequently, an artificial compound could be produced during homogenizing or chromatography. However, we had previously succeeded in excluding this possibility; vanadobin could be extracted from a homogenate of a pure subpopulation of signet ring cells (the vanadocyte), but not from that of the morula cells. In these experiments we used a combination of cell fractionation for purification of a specific type of blood cell, chromatography for extraction of vanadobin, and neutron activation analysis for determination of vanadium (Michibata and Uyama, 1990).

The present results clearly indicate that vanadobin is contained in the blood cells of all the ascidians examined. In general, ascidians belonging to the family Ascidiidae contain high levels of vanadium, whereas those belonging to Cionidae (Michibata, 1984; Michibata *et al.*, 1986b) and Corellidae (Hawkins *et al.*, 1983) do not always have such high amounts of vanadium. The amount of vanadium, even in vanadium-poor ascidians, is, however, thousands of times higher than that in other animals, including mammals. We may suppose, therefore, that other kinds of complexes with vanadium must be present in ascidian tissues. The occurrence of vanadobin in the blood cells of *Ciona intestinalis* and *Corella japonica* suggests that vanadobin is a universal complex in ascidian blood cells, and that its role is to accumulate and maintain vanadium.

One of the reasons that previous attempts to obtain vanadium-binding substances failed might be that the pH of the buffer solution used in the extraction of vanadium-binding substances was neutral. (There is utter confusion about the actual intracellular pH of ascidian blood cells.) Following Henze's first discovery of 1 *N* acidity (Henze, 1911), it was widely accepted that a homogenate of ascidian blood cells had a low pH value. However, Dingley *et al.* (1982) and Agudelo *et al.* (1983) claimed that the methods used in those early experiments gave spurious results because the cell interior containing the high levels of vanadium was most probably a highly reducing environment. Therefore, the possibility has not been eliminated that the intracellular redox potential, not the pH, was measured. When a new technique with an improved trans-membrane equilibrium of ^{14}C-labeled methylamine was used, the intracellular pH was neutral. Hawkins's group also measured nearly neutral pH in ascidian blood cells by means of a ^{31}P-NMR (Hawkins *et al.*, 1983; Brand *et al.*, 1987). Conversely, Frank *et al.* (1986) reported that the blood cells possess a pH value of 1.8 based on ESR spectrometry. These previous studies were, however, carried out on whole blood cells, without cell fractionation, and were focused on the green-hued morula cells which had been considered vanadocytes; not examined were the signet ring cells that have been newly identified as vanadocytes (Michibata *et al.*, 1987). In fact, we have recently demonstrated that the separated subpopulations of signet ring cells in *Ascidia ahodori, A. sydneiensis samea, A. gemmata,* and *Corella japonica* show low pH values ranging from 0.8 to 3.1, and that they also contain high amounts of vanadium, as determined by a combination of techniques involving cell fractionation, microelectrode measurements, and neutron activation analysis (Michibata *et al.*, 1990b). From this angle, it seems highly possible that vanadium binds with organic substances within the signet ring cells (strictly speaking, in the vacuole), and therefore, the previous failures in the extracting vanadium-binding substances were due to conditions of pH. We also could not extract vanadobin under neutral conditions (data are not shown).

Roman *et al.* (1988) have recently extracted metal complexes with organic substances from the blood plasma of *Pyura chilensis* and *Ascidia dispar.* These substances seem to be heavier molecules than vanadobin because they eluted through Sephadex G-75. The blood plasma has been proposed to contain transferrin-like metalloprotein, although the biochemical roles in ascidian blood are still unknown (Roman *et al.*, 1988). On the other hand, because vanadobin is estimated to be a low molecular weight substance (about 1300), and the concentration of vanadium in the blood cells is 100 or more times greater than that in the blood plasma (Michibata *et al.*, 1986b; Roman *et al.*, 1988), these two types of metal binding substances probably have different roles in ascidian blood.

The highest concentration of vanadium was contained in the blood cells of *A. gemmata;* this corresponds to 4,000,000 times that of seawater (*cf.* Michibata, 1989). Strictly speaking, if almost all of the vanadium observed in the blood cells is contained in the vacuoles and binds with the vacuole membranes (Scippa *et al.*, 1988), its concentration should be much higher. Vanadobin, which can maintain the vanadium ion in the reduced form of +3 or +4 and has an affinity for exogenous vanadium ion (Michibata *et al.*, 1986a), must hold the key to resolving the specific accumulation of vanadium. Therefore, the next important study is to clarify the chemical structure of vanadobin.

Acknowledgments

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Diffusion Limitation and Hyperoxic Enhancement of Oxygen Consumption in Zooxanthellate Sea Anemones, Zoanthids, and Corals[1]

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Abstract. Depending on their size and morphology, anthozoan polyps and colonies may be diffusion-limited in their oxygen consumption, even under well-stirred, air-saturated conditions. This is indicated by an enhancement of oxygen consumption under steady-state hyperoxic conditions that simulate the levels of O_2 produced photosynthetically by zooxanthellae in the hosts' tissues. Such hyperoxia in the tissues of zooxanthellate species negates the effect of the diffusive boundary layer, and increases the rate of oxygen consumption; thus, in many cases, the rate of respiration measured under normoxia in the dark may not be representative of the rate during the day when the zooxanthellae are photosynthesizing and when the supply of oxygen for respiration is in the tissues themselves, not from the environment. These results have implications in respirometric methodology and in calculating the rate of gross photosynthesis in energetic studies. The activity of cytochrome *c* oxidase is higher in aposymbiotic than in zooxanthellate specimens of the sea anemone *Aiptasia pulchella*, and this may indicate a compensation for the relative hypoxia in the tissues of the former, enhancing the delivery of oxygen to the mitochondria from the environment.

> "... [*zooxanthellae*] *certainly provide abundant supplies of oxygen, without which it is* just *possible that such immense aggregations of living matter which constitute a coral reef... could not originate and flourish.*"
>
> —*C. M. Yonge (1930)*

Introduction

The relatively weak oxyregulatory ability apparent in most anthozoans stems in part from their scant ability to create bulk flow in the seawater surrounding them. In the laboratory, therefore, oxyregulation depends on the amount of convection provided by the experimental apparatus. Under well-stirred conditions, the diffusive boundary layer at the body surface will be thin, and turbulence especially will reduce diffusion gradients. At lower current speeds, the boundary layer thickens, and oxygen uptake becomes more diffusion-limited; this effect is pronounced at low-to-intermediate oxygen partial pressures, so that there is a marked effect of convection on apparent oxyregulatory ability (see Dromgoole, 1978).

The earliest studies of the effect of oxygen partial pressure (P_{O_2}) on the rate of oxygen consumption in anthozoans were made with little or no stirring of the medium, and indicated little or no oxyregulation by *Actinia equina, Anemonia viridis* (=*A. sulcata*) (Henze, 1910), several scleractinian corals (Yonge *et al.*, 1932), *Calliactis parasitica,* and several pennatulids (Brafield and Chapman, 1965). Under well-stirred conditions, the rate of oxygen consumption by most anthozoans increases curvilinearly with P_{O_2}. Recently obtained curves are hyperbolic, and most approach an asymptote at air saturation (*cf.* Mangum and Van Winkle, 1973; Sassaman and Mangum, 1972, 1973, 1974; Shumway, 1978; Ellington, 1982; Tytler and Davies, 1984). In such studies, the rate is often explicitly or implicitly assumed to reach a plateau at air saturation (20.95% O_2, corresponding to 21.23 kPa at one atmosphere), although Mangum and Van Winkle (1973) emphasized that the assumption of a plateau in the commonly used hyperbolic model is not always realized in the data. The assumption has not been

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tested in anthozoans, except by Henze's (1910) experiments, where a hyperoxic enhancement of oxygen consumption is evident, albeit under apparently unstirred conditions.

Similarly, investigators of coral productivity assume that respiration proceeds at the same rate in daylight and at night. But when the illuminated zooxanthellae in symbiotic anthozoans are photosynthesizing, oxygen levels in their tissues rise well above air saturation (D'Aoust *et al.*, 1976; Crossland and Barnes, 1977; Dykens and Shick, 1982). Most concerns about the high oxygen levels in the tissues have centered on possible photorespiration or inhibition of photosynthesis in the zooxanthellae (Black *et al.*, 1976; Downton *et al.*, 1976), or on potential oxygen toxicity in the host (D'Aoust *et al.*, 1976; Dykens and Shick, 1982; Shick and Dykens, 1985) and zooxanthellae (Lesser and Shick, 1989).

A role for the zooxanthellae as endogenous providers of oxygen to the host during environmental hypoxia was shown by Shick and Brown (1977), following the demonstration that zooxanthellae affect the spacing between clonal anemones (Fredericks, 1976). Moreover, increased convection in the air-saturated medium increases the rate of oxygen consumption in sea anemones, octocorals, and scleractinian corals (Dennison and Barnes, 1988; Patterson and Sebens, 1989), which implies that a diffusive boundary layer exists and that it impedes the delivery of oxygen to the tissues, even under well-oxygenated conditions. Thus, depending on the extent of external convection—which itself may vary within a coral colony, depending on its hydrodynamic porosity (Chamberlain and Graus, 1975)—oxygen generated photosynthetically *within* the tissues might well negate the effects of the boundary layer and elevate respiration above that measured in darkness at air saturation.

The present paper reports the effects of hyperoxia on oxygen consumption in zooxanthellate anthozoans; the degree of hyperoxia used is within the range known to occur in the tissues of the animals (see Dykens and Shick, 1982). Experimental subjects were chosen to exemplify a range of the size and morphological complexity of polyps, and the growth form of colonies. Finally, the maximum activities of cytochrome *c* oxidase are presented for zooxanthellate and aposymbiotic (lacking zooxanthellae) specimens of *Aiptasia pulchella* maintained under different levels of illumination and oxygenation.

Materials and Methods

Specimens of the sea anemone *Aiptasia pallida* and the zoanthid *Zoanthus sociatus* were collected in the vicinity of the Bermuda Biological Station and were maintained in the station's seawater system prior to use in experiments. Steady-state measurements of oxygen consumption in the dark were made in a BioMetric-CYCLOBIOS twin-flow microrespirometer fitted with Orbisphere model 2120 polarographic oxygen sensors. Millipore-filtered (0.45 μm pore size) seawater (37‰S, 25°C) entering the 50 cm^3 animal chamber was equilibrated sequentially with O_2:N_2 mixtures of 21%:79% (normoxia), 50%:50% (hyperoxia), normoxia again, and 10%:90% (hypoxia) using Tylan FC-260 mass-flow controllers. Perfusion of the animal chamber via an LKB MicroPerpex peristaltic pump was varied between 25 and 75 cm^3 h^{-1}, to maintain an oxygen reduction ratio (see Gnaiger, 1983) of about 2–6% between the sensors measuring the oxygenation of seawater entering and leaving the chamber. Water in the chamber was well mixed with a magnetic stirrer situated beneath a perforated plate to which the specimen was attached. Stirring speed was 200 rpm, the maximum that could be used without causing the anemone to collapse or contract. Measurements at each oxygen level were continued for at least 4 h. Values were corrected for blank oxygen consumption at each P_{O_2}.

In a subsequent experiment, a specimen of *Aiptasia pallida* was placed in the 3.5 cm^3 perfusion cell of a ThermoMetric 2277 Thermal Activity Monitor in series with the twin-flow microrespirometer, both regulated at 20°C. The chamber was perfused at a flow rate of 27 cm^{3}h^{-1} for 9 h with 30‰S seawater equilibrated with 21% O_2: 79% N_2, and for an additional 4 h with seawater equilibrated with 50% O_2:50% N_2. Simultaneous fluxes of metabolic heat and oxygen were continuously monitored and analyzed as described in Gnaiger *et al.* (1989).

The clownfish sea anemone *Heteractis crispa*, colonies of the zoanthids *Palythoa tuberculosa* and an unidentified species of *Protopalythoa*, and the scleractinian coral *Stylophora pistillata* were collected from Davies Reef on the Great Barrier Reef, Australia. The sea anemone *Phyllodiscus semoni* was taken from the fouling community in the seawater system at the Australian Institute of Marine Science. Oxygen consumption by *H. crispa*, by the zoanthids and coral, and by large specimens of *P. semoni* was measured in the dark in a closed respirometer (2.3 dm^3) fitted with a Radiometer E5046 oxygen sensor connected to a Radiometer PHM72 Mk2 acid-base analyzer. The specimen was placed on a perforated platform above a large magnetic stirrer operated continuously at 500 rpm, the highest speed that did not disturb *P. semoni*. Polyps of the colonial anthozoans generally remained expanded under this stirring regime. Each specimen was placed in the respirometry vessel, and the seawater (32‰–34‰S, 30°C) bathing it was bubbled with a mixture of 55% O_2:45% N_2 delivered by a Wösthoff type SA/18 gas mixing pump for at least one hour before the chamber was sealed and measurement of oxygen consumption begun. This equilibration period was intended to eliminate transient, diffusional redistribution of oxygen in which *uptake* of oxygen by the relatively hypoxic body fluids or skeletal pore water of a specimen transferred acutely from the tanks of air-saturated seawater to the respirometer might be interpreted as an initially high rate of *consumption* of oxygen (see Dromgoole, 1978). During the measurements, the specimen was allowed to deplete the oxygen in the respirometer to just below air saturation; the chamber was then flushed, reequilibrated with 55% O_2 for one hour, and the measurements repeated. In cases where the two measurements of oxygen consumption over a particular range of P_{O_2} (either hyperoxia—46.7–45.4 kPa, or normoxia—21.2–20.0 kPa) did not agree to within ±10%, the experiment was performed a third time, and the rates of oxygen consumption at both oxygen levels were calculated as the mean of the three measurements at each level. Respiration rates at each oxygen level were corrected for the blank which, in the case of *Protopalythoa* sp., included oxygen uptake by the substrate from which the polyps were removed after the experiment.

The mass of the specimens was variously measured at the end of the respiration experiments, the particular measure being largely a matter of convenience. For *P. tuberculosa* and *Protopalythoa* sp., blotted wet weight ($_wW$) was used, whereas freeze-dried weight ($_dW$) was used for *Z. sociatus* and *P. semoni.* The protein content in individual *A. pallida* was measured by the microbiuret method with bovine serum albumin standards, and in whole colonies of *S. pistillata* by the Bio-Rad Coomassie dye-binding method with bovine gamma globulin standards. As an index of the hydrodynamic porosity of colonies of *S. pistillata,* the ratio of the mean distance between nearest neighbor branches, to the mean branch diameter, was calculated (Chamberlain and Graus, 1975).

Clonal cultures of *Aiptasia pulchella* (obtained from L. Muscatine, University of California, Los Angeles) were maintained in artificial seawater (Instant Ocean, 30‰S) at 25°C. Groups of zooxanthellate anemones were exposed to irradiances of 85 (Dim) or 420 (Bright) μmol $m^{-2}s^{-1}$ under the beam of a Kratos SS1000X 1 kW xenon arc solar simulator (air mass 1 filter). One culture of aposymbiotic anemones (Apo) was maintained in continuous darkness in air-saturated seawater, and another group of aposymbiotic specimens was maintained in the dark in seawater continuously bubbled with 50% O_2 (ApoHiO_2). After two weeks of acclimation to these conditions, individual anemones were homogenized (10% w/v) in 100 m*M* potassium phosphate buffer (pH 7.0). Homogenates were centrifuged at 500 × *g* for 20 min to remove intact zooxanthellae and animal debris, and cytochrome *c* oxidase (EC 1.9.3.1) in the animal supernatant was assayed at 25°C according to the modified method of Hansen and Sidell (1983), using reduced cytochrome *c* (Sigma Type III).

Results

Specific rates of oxygen consumption (μmol O_2 $g^{-1}h^{-1}$ on the basis of wet or dry weight, or nmol O_2 mg protein^{-1} h^{-1}) by the several species under various oxygen regimes are given in Figures 1–4. Interspecific comparisons of specific rates are not meaningful in the present experiments, owing to the different measurements of mass, which are further complicated by the variable amounts of inorganic material (*e.g.*, sand) in the coenenchyme of the zoanthids.

The rate of oxygen consumption in *Aiptasia pallida* increased at a partial pressure of oxygen approximately twice air saturation (Fig. 1). Although slight, the 11% enhancement of respiration at 50% O_2 is statistically significant (paired $t = 4.04$, df = 4, $P = 0.016$). This is because most of the variance seen in Figure 1 occurred between specimens; all individual anemones showed higher rates of oxygen consumption at 50% than at 21% O_2, which accounts for the significance seen in the paired *t*-test. The direct calorimetric experiment confirmed that the elevated rate of oxygen uptake reflected an increase in aerobic energy metabolism, as the steady rate of heat dissipation by the anemone increased from 30 μW at 21% O_2 to 33 μW at 50% O_2 (Fig. 1, inset), a 10% rise that closely matched the independent respirometric results. When rates of both heat dissipation and oxygen consumption were steady, the calorimetric-respirometric (CR) ratio was 0.451 μJ pmol^{-1} O_2 at 21% O_2, and 0.463 μJ pmol^{-1} O_2 at 50% O_2. Neither of these values differs significantly from the theoretical oxycaloric equivalent of 0.450 μJ pmol^{-1} O_2 for fully aerobic metabolism (Gnaiger *et al.*, 1989).

The much greater (43%) enhancement, by hyperoxia, of respiration in *Phyllodiscus semoni* (Fig. 2A) is likewise highly significant (paired $t = 6.25$, df = 3, $P = 0.008$). The rate of oxygen consumption in the specimen of *Heteractis crispa* increased by 26% during hyperoxia (Fig. 2B).

Sample sizes for the zoanthids are small (only one or two specimens of each species). Recall, however, that there are two or three measurements (continuous measurements over four hours, in the case of *Zoanthus sociatus*) of oxygen consumption in each species at each oxygen level, so that although this pseudoreplication does not permit statistical analysis, any observed difference in oxygen consumption with P_{O_2} is real. Morphologies of the three species are shown in Figure 3, together with the data. Hyperoxic enhancement of respiration in *Palythoa tuberculosa* averaged 46% (79% in one colony and 23% in a second), whereas the average of repeated measurements on one colony of *Protopalythoa* sp. indicated a slight (8.6%) decline at 50% O_2. Long-term measurements on *Z. sociatus* revealed a 21% increase in a colony of five closely spaced individuals, but essentially no effect (3% increase) of hyperoxia on oxygen consumption in a single polyp subsequently isolated from the colony.

Data are presented separately for two ecomorphs of the scleractinian coral *Stylophora pistillata,* shown in

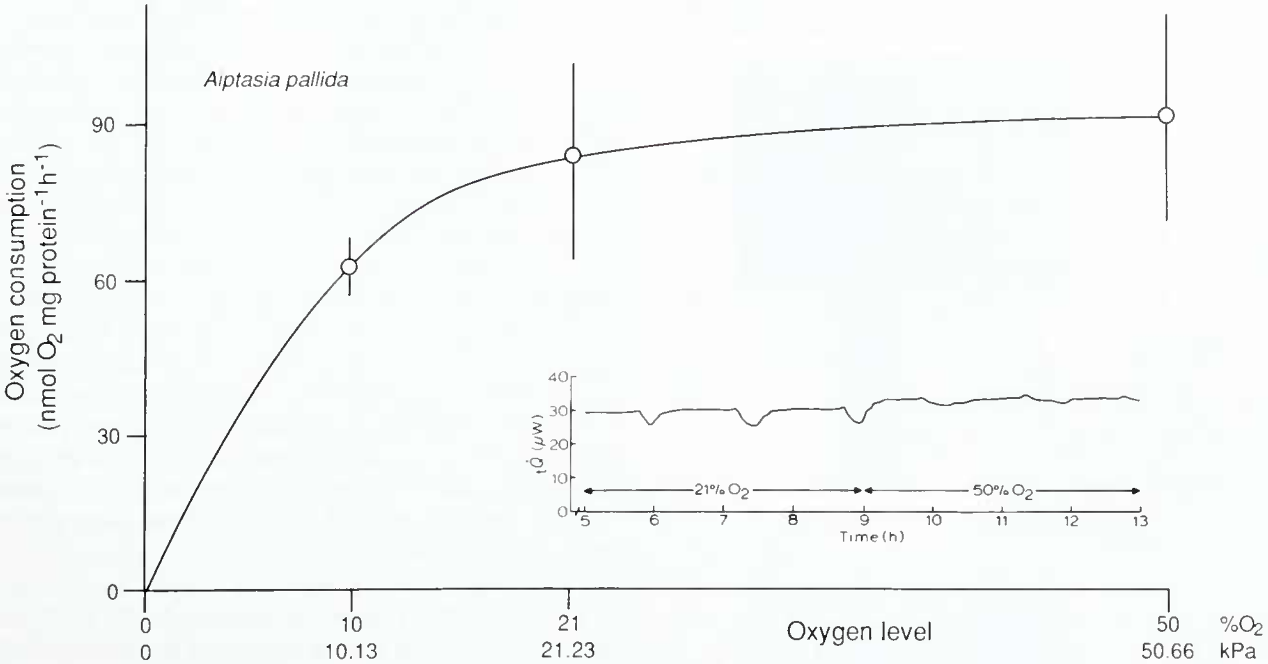

Figure 1. Rates of oxygen consumption in *Aiptasia pallida* (n = 5; mean size = 2.862 mg protein, range = 1.37–4.18 mg protein) under conditions of hypoxia (10% O_2 at inflow to respirometer), normoxia (21% O_2), and hyperoxia (50% O_2). Vertical lines indicate ±1 standard error. Inset: instantaneous heat flux ($_t\dot{Q}$, μW) in a specimen of *A. pallida* (0.788 mg protein) exposed to 21% O_2 and 50% O_2 in an open-flow calorimeter.

Figure 4. Two colonies of the ecomorph having thin, widely spaced branches consistently showed a slight (9.0% and 7.5%) decrease in respiratory rate under hyperoxia, whereas three colonies having thick, closely spaced branches showed a mean 20% hyperoxic enhancement of respiration that was significant (paired t = 5.69, df = 2, P = 0.030). The magnitude of the effect of hyperoxia on oxygen consumption in *S. pistillata* seems to be inversely related to the hydrodynamic porosity of the colony (Fig. 5).

Rates of cytochrome *c* oxidase activity in the various groups of *Aiptasia pulchella* are shown in Figure 6. Specific activity is expressed in Units per mg protein in the supernatant, each Unit corresponding to 1 μmol cytochrome *c* oxidized per minute. Analysis of variance indicated a significant effect of treatment on enzymatic activity (F = 7.942, df = 3, 16, P = 0.0018). Individual means were compared using the Student-Newman-Keuls test with a significance level of 0.05.

Discussion

The available studies of effects of hyperoxia on the rate of oxygen consumption in fishes and aquatic invertebrates indicate no enhancement (and perhaps a slight reduction) of the rate relative to that under normoxia. This is largely due to decreases in the ventilatory convection requirement under hyperoxia (Dejours and Beekenkamp, 1977; Toulmond and Tchernigovtzeff, 1984; Berschick *et al.*, 1987), and to the presence of respiratory pigments, both of which stabilize the delivery of oxygen to the tissues over a wide range of external P_{O_2}. Lacking respiratory pigments, and having only weak powers of convection of the external medium, cnidarians are more at the mercy of the Fick equations for diffusive gas exchange. As such, their respiratory exchange must be markedly affected by the flow regime they occupy, and in the case of species harboring algae, by provision of O_2 from those photosynthetic symbionts.

Many anthozoans, especially sea anemones, show behavioral compensations for varying levels of water movement and environmental oxygen supply. A positive relationship exists between the degree of inflation of the hydrostatic skeleton and current velocity in *Metridium senile* (Robbins and Shick, 1980). Although this seems primarily related to prey capture in this suspension feeder, full extension of the column and tentacles also simultaneously maximizes the surface-to-mass ratio and minimizes diffusion distances within the tissues, and thus maximizes oxygen delivery and hence the rate of oxygen consumption (see Shick *et al.*, 1979).

Behaviors that increase the surface area and decrease diffusion distances in the primary gas exchange surfaces are also seen as adaptive short-term responses to hypoxia

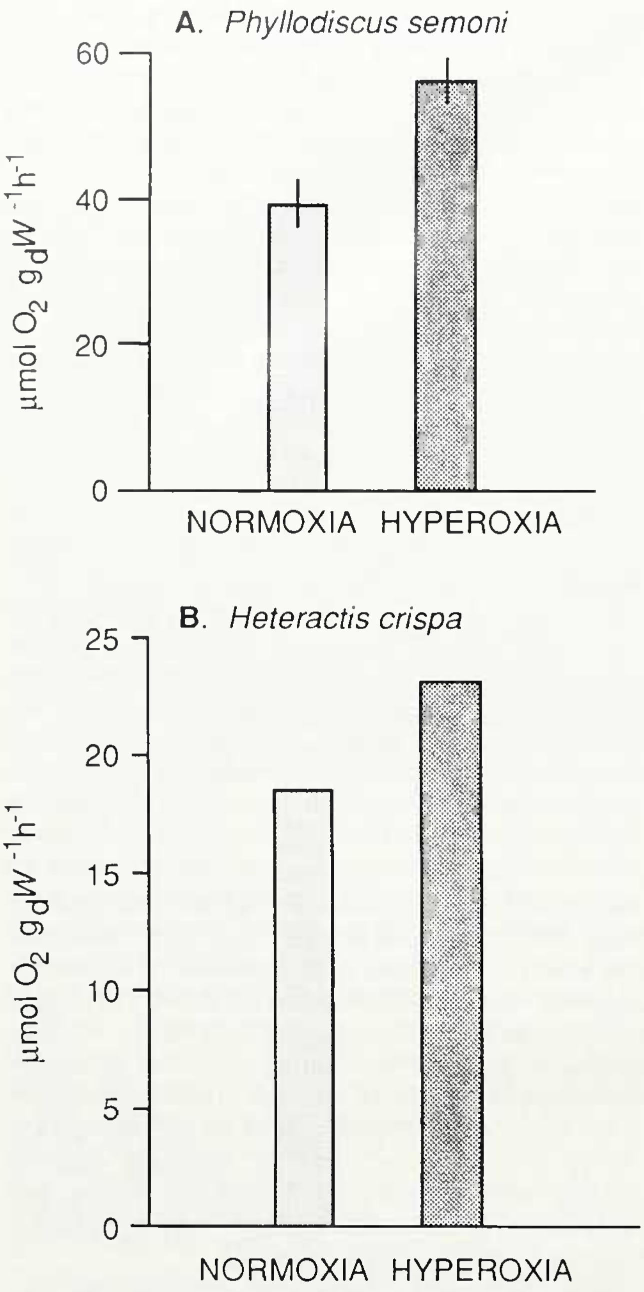

Figure 2. (A) Rates of oxygen consumption in *Phyllodiscus semoni* (n = 4; mean $_dW$ = 0.661 g, range = 0.599–0.725 g) under normoxia (20.0–21.2 kPa O_2) and hyperoxia (45.4–46.7 kPa O_2). Vertical lines indicate ±1 standard error. (B) Rates of oxygen consumption in a 2.898 g $_dW$ aposymbiotic specimen of *Heteractis crispa* under normoxia and hyperoxia.

in several species of sea anemones and ceriantharians (Sassaman and Mangum, 1972, 1974; Shick *et al.*, 1979). Conversely, the zooxanthellate sea anemones *Anthopleura elegantissima* and *A. xanthogrammica* contract under peak levels of solar irradiance, a response that seems related more to avoiding the damaging photodynamic effects of interacting ultraviolet radiation and hyperoxia, than to hyperoxia *per se* (Shick and Dykens, 1984). In the absence of UV, *A. elegantissima* remains expanded under moderate levels of irradiance that yield a net production of oxygen (and hence, tissue hyperoxia) by its zooxanthellae (Shick and Brown, 1977).

Earlier studies on anthozoans centered on the respiratory response to hypoxia and did not involve oxygen levels above air saturation. This is understandable, because most of the species that were studied do not contain zooxanthellae and would not experience hyperoxia, except perhaps in tidepools where free-living algae might produce transient hyperoxia (see Truchot and Duhamel-Jouve, 1980). Nevertheless, inspection of published curves (see references in Introduction) suggests that, in most cases, an increase in respiration would occur under hyperoxia, and this has relevance particularly in zooxanthellate species that routinely experience such oxygen levels in their tissues.

The hyperoxic enhancement of oxygen consumption in *Aiptasia pallida* reported here is admittedly slight, but has consequences in the calculations of budgets of energy and carbon in the symbiosis. Specifically, because respiration under 50% O_2 (a level that occurs in the tissues of symbiotic anthozoans when their zooxanthellae are photosynthesizing: D'Aoust *et al.*, 1976; Dykens and Shick, 1982) is 11% higher than at normoxia, daytime respiration is underestimated when respiration is measured at air saturation. Because the compensation irradiance (where respiration is balanced by photosynthesis) is less than 50 μmol $m^{-2}s^{-1}$, and the saturation irradiance for photosynthesis in the zooxanthellae is 200–400 μmol $m^{-2}s^{-1}$ in *Aiptasia* spp. (Muller-Parker, 1984; Lesser and Shick, 1989), and considering that irradiances in the habitats of *Aiptasia* spp. exceed 100 μmol $m^{-2}s^{-1}$ for most of the daylight period (Muller-Parker, 1987; Lesser and Shick, unpubl. data), there is a net production of oxygen for most of the day. Thus, 50% O_2 is a realistic level of tissue oxygenation for this period (also see D'Aoust *et al.*, 1976). Therefore, daytime respiration in *Aiptasia* spp. has routinely been underestimated by ≈11%; the apparent value of net photosynthesis is therefore misleading, and neglect of this would underestimate gross photosynthesis by a corresponding amount.

In the natural habitat of *A. pallida* at Walsingham Pond, Bermuda, the diurnal increase in respiration owing to hyperoxia is slightly offset each night, caused by a brief decline in seawater oxygenation to one-half or even one-third of air saturation (K. Eakins, pers. comm.). Inspection of Figure 1 indicates that this would result in about a 33% decline in respiration, for a period of about 3 h (Eakins, *loc. cit.*).

Among the species studied here, *Aiptasia pallida* would be the least likely to be diffusion-limited in its respiratory gas exchange, owing to its small size and morphological simplicity. In the sea anemones *Phyllodiscus*

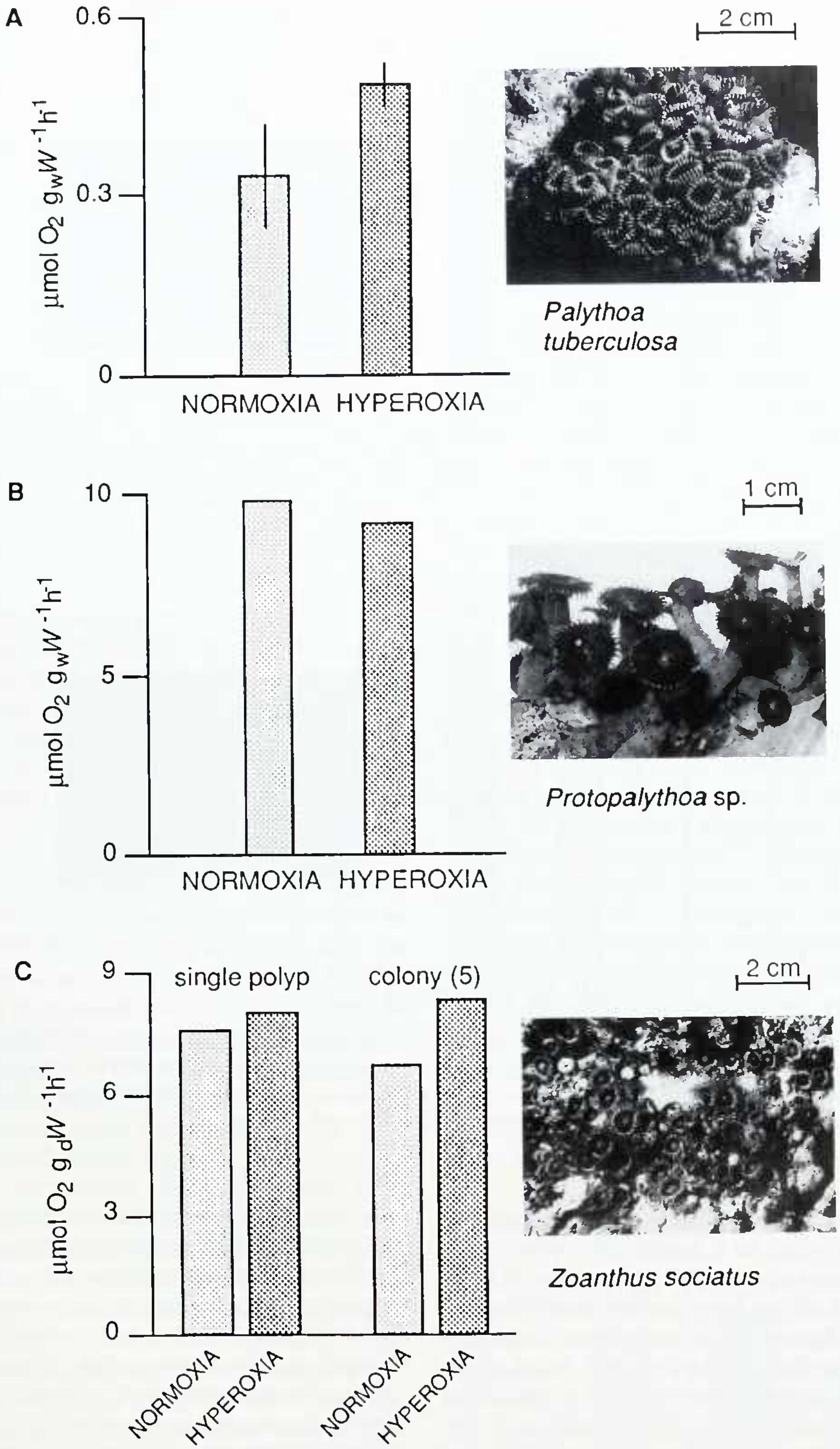

Figure 3. Rates of oxygen consumption in zoanthids. In colonies of (A) *Palythoa tuberculosa* (mean $_wW$ = 47.25 g) and (B) *Protopalythoa* sp. ($_wW$ = 2.89 g), normoxia corresponds to 20.0–21.2 kPa O_2 and hyperoxia to 45.4–46.7 kPa O_2. Vertical lines in (A) indicate ±1 standard error. In *Zoanthus sociatus* (C), the group of 5 individuals weighed 0.011 g $_dW$ and the single polyp 0.003 g $_dW$. Oxygen levels at the inflow to the respirometer were normoxia (21% O_2) and hyperoxia (50% O_2).

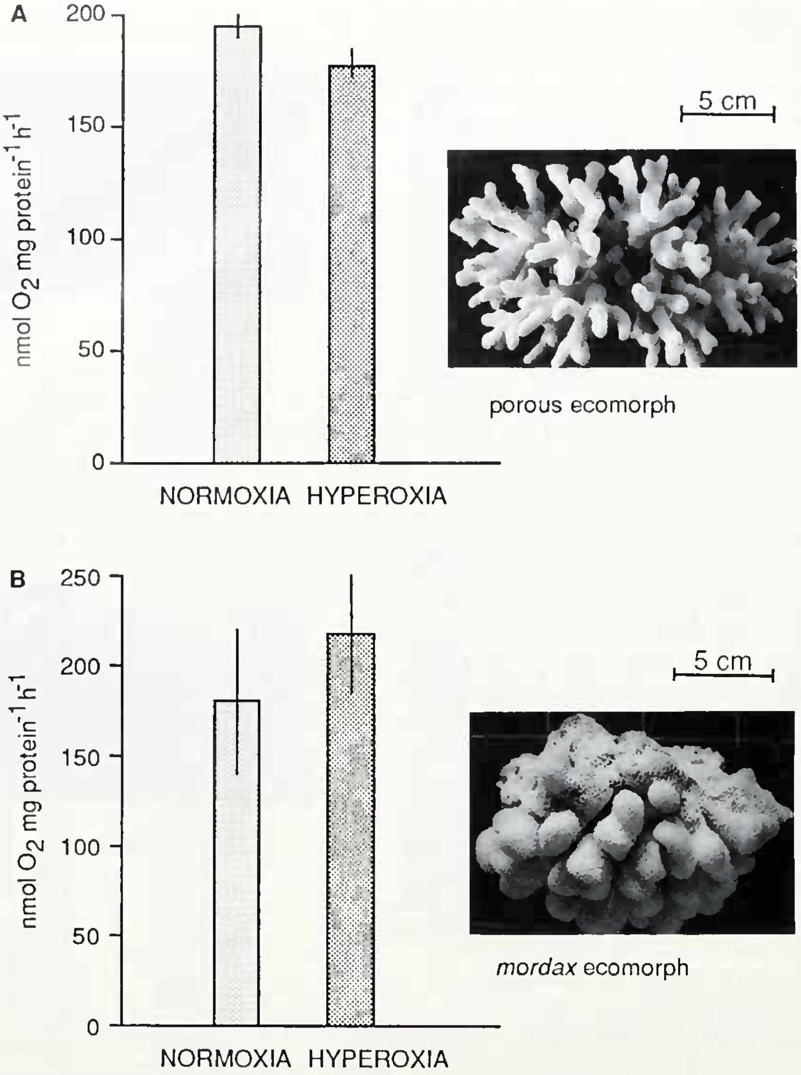

Figure 4. Rates of oxygen consumption in colonies of *Stylophora pistillata* under normoxia (20.0–21.2 kPa O_2) and hyperoxia (45.4–46.7 kPa O_2). (A) In the hydrodynamically porous ecomorph (n = 2), average total colony protein was 369 mg. (B) In the *mordax* ecomorph (n = 3), mean total colony protein was 586 mg. Vertical lines indicate ±1 standard error. Photographs by J.-P. Gattuso.

semoni and *Heteractis crispa,* hyperoxia increases the respiratory rate more than in *A. pallida.* This seems to be related to their much larger size (5 to 10 cm, *vs.* 0.5 cm diameter) and perhaps to their greater morphological complexity. These factors might increase the boundary layer at the body surface, in the first case, because the thickness of the boundary layer around a cylindrical anemone is related to the square root of its diameter (Vogel, 1983). In addition, water flow might be impeded among the numerous pseudotentacles in *P. semoni* and tentacles covering the oral disc of *H. crispa.* Such additional roughness elements may also increase eddy currents and increase the residence time of water in the boundary layer. The existence of such a boundary layer under conditions of forced convection is documented by the experiments of Patterson and Sebens (1989), who found that oxygen consumption by specimens of *Metridium senile* of about the same size as the *Phyllodiscus* in the present study increased two- to threefold as current speed increased from ≈7 to 15 cm s^{-1}. Therefore, endogenously produced oxygen (which need not negotiate an external boundary layer) is proportionally more important to large anemones that experience a larger boundary layer that develops even under well-stirred conditions, than to small anemones. Measurements of oxygen flux in large zooxanthellate anemones under normoxic conditions will accordingly underestimate their daytime respiration (and so underestimate gross photosynthesis) more than in small anemones.

Unlike unitary anemones, zoanthids form colonies of

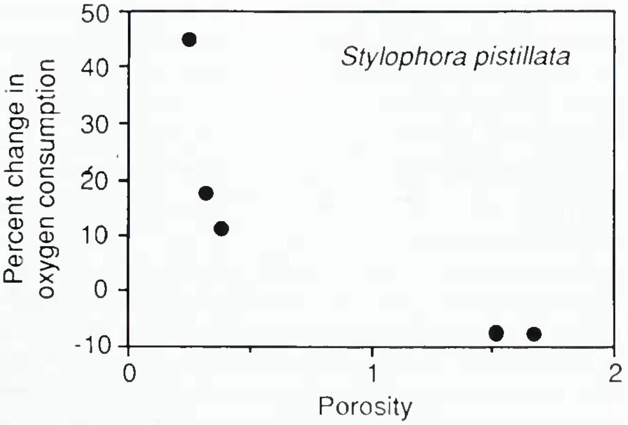

Figure 5. Relationship between the hyperoxia-induced change in oxygen consumption and hydrodynamic porosity in colonies of *Stylophora pistillata.* Porosity was calculated as the ratio of the mean distance between nearest neighbor branches to mean branch diameter. Each point represents one colony.

interconnected polyps that vary in their spacing and aggregate morphology, and thus in their hydrodynamic properties. Such variation has been discussed primarily with respect to the provision of food to these sessile filter feeders (*e.g.*, Koehl, 1977), but the hydrodynamic principles apply to the delivery of O_2 as well. At one morphological extreme, *Palythoa tuberculosa* forms platelike colonies of closely conjoined polyps embedded in a massive, largely inorganic, coenenchyme. Water flow across the colony decreases with increasing distance from the periphery, as kinetic energy is extracted from the flow by skin friction and by form drag of the polyps; thus the interior polyps experience relatively stagnant conditions compared to their clonemates on the edge. Delivery of oxygen to respiring tissues is also impaired by the protective coenenchyme. At the other extreme, *Protopalythoa* sp. from Australia forms loosely aggregated colonies of tall, widely separated polyps connected only at their bases and having less coenenchyme.

Accordingly, *P. tuberculosa* might be expected to be more diffusion-limited in its gas exchange, whereas *Protopalythoa* sp. would perform more like individual small anemones. These predictions are confirmed experimentally, because hyperoxia generates increases in oxygen consumption in *P. tuberculosa* but not in *Protopalythoa* sp. (*cf.* Fig. 3A and B). Moreover, a group of closely spaced polyps of *Zoanthus sociatus* shows hyperoxic enhancement of respiration, whereas a single small polyp responds more like a solitary *Aiptasia pallida* and shows minimal enhancement (Fig. 3C). The difference between individual and colonial respiratory performance in the last case seems to be related to a decrease in free surface area and restriction of water flow between polyps, and hence to a larger effective diameter, with the resultant decrease in water flow toward the center of the colony.

Previous studies concerning physiological effects of water flow around scleractinian coral colonies have been focused primarily on provision of food and removal of waste, although Jokiel (1978) suggested that respiratory exchange is also affected. Dennison and Barnes (1988) and Patterson *et al.* (1990) subsequently demonstrated that respiration under normoxic conditions in *Acropora formosa* and *Montastrea annularis* does increase in moving water. More to the present point, enhancement by light of calcification in *Stylophora pistillata* was suggested by Rinkevich and Loya (1984) to be due to stimulation of (aerobic) metabolism by O_2 produced within the symbiosis by the zooxanthellae. Together these studies indicate that a boundary layer to the delivery of oxygen exists even under normoxic conditions, and that under some circumstances its effects are negated by the production of oxygen within the host's tissues.

Stylophora pistillata exhibits a particularly great diversity of colonial morphologies, to a large extent determined by the flow regime where it occurs (Veron and Pichon, 1976), as well as by photic regime (McCloskey and Muscatine, 1984; Titlyanov, 1987). Chamberlain and Graus (1975) conclude that flow within a branching colony depends entirely on its morphology and on exterior hydrodynamic conditions. Therefore, the prevalence of colonies of *S. pistillata* having thin, widely spaced branches in low energy habitats (Veron and Pichon, 1976; pers. obs.) seems related to the maintenance of adequate flow among the branches in such sheltered areas (and to the avoidance of self-shading in deep water or shaded sites). Although the thick, closely spaced branches of the *mordax* ecomorph of this species would seemingly restrict flow to the interior of the colony, this ecomorph inhabits high energy environments (Veron and Pichon, 1976; pers. obs.). Thus, the ecomorphs of *S. pistillata* exemplify the principle of dynamic similitude—under the appropriate flow conditions, morphologically dissimilar colonies can have similar flow characteristics (Chamberlain and Graus, 1975) and respira-

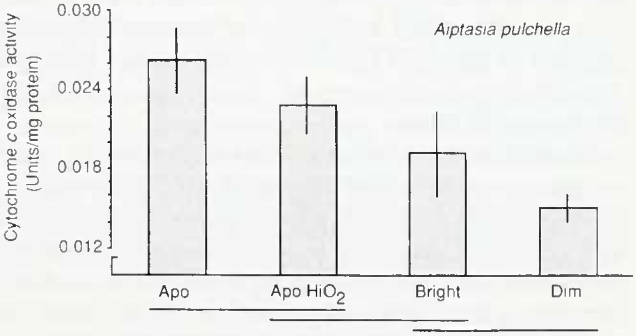

Figure 6. Cytochrome *c* oxidase activity in aposymbiotic and zooxanthellate specimens of *Aiptasia pulchella* (n = 5 in each treatment). Vertical lines indicate ±1 standard error. Horizontal lines underscore groups whose means are not significantly different ($P > 0.05$).

tory rates. The corallites in this species are relatively shallow, so that diffusion distances within the corallum and tissues are short, and probably similar in the different ecomorphs.

In the moderate, turbulent flow in the respirometer, colonies of the hydrodynamically porous ecomorph experience no diffusion limitation, as hyperoxia does not result in an increase in oxygen consumption (Fig. 4A). Similar results are obtained with single branches of colonies (Shick, unpub. data; J.-P. Gattuso, pers. comm.), in which water flow and delivery of oxygen to the polyps is not hindered by any nearby branches. Colonies of the high-energy *mordax* ecomorph, however, do appear to be diffusion limited even under these well-mixed conditions, showing a 20% hyperoxic increase in respiration (Fig. 4B), probably owing to elevated O_2 levels among the interior branches of the colonies. Consequently, the magnitude of the effect of hyperoxia on respiration is inversely related to the hydrodynamic porosity of the colony under these conditions (Fig. 5). Whether this diffusion limitation is more pronounced under unidirectional, laminar flow, or under the turbulent conditions in the present study is unknown, but this is testable with a flow tunnel respirometer.

Compensation for different levels of oxygenation may also be manifested at the cellular level. Lacking zooxanthellae, the tissues of aposymbiotic anemones are hypoxic relative to those of zooxanthellate conspecifics when the latter are photosynthesizing. In *Aiptasia pulchella*, this is associated with a significantly higher activity of cytochrome *c* oxidase (the terminal enzyme in the mitochondrial respiratory chain) in aposymbiotic than in zooxanthellate clonemates. Such an elevation of mitochondrial respiratory capacity in the relatively hypoxic anemones could result from more or larger mitochondria, greater specific activity of cytochrome *c* oxidase per mitochondrion, or a combination of these. Stereological studies of the numbers, distribution, and ultrastructure of mitochondria in anemones under these conditions are in progress. Increasing the numbers of mitochondria (and hence reducing the diffusion distance for oxygen from the cell surface to a respiring mitochondrion) is a correlate of intertidal hypoxic exposure in the anemone *Anthopleura elegantissima* (J. A. Dykens and J. M. Shick, unpubl. data).

The data on aposymbiotic *A. pulchella* cultured under exogenous hyperoxia are consistent with this postulate. Its cytochrome *c* oxidase activity is intermediate to the high activity in relatively hypoxic aposymbiotic clonemates and to the low values in its hyperoxic zooxanthellate clonemates; in the latter case, the major source of oxygen for much of the time is endogenous, from the intracellular zooxanthellae. The lack of effect of irradiance (Dim *vs.* Bright) on cytochrome *c* oxidase activity in zooxanthellate anemones suggests that both groups (maintained at irradiances exceeding the compensation point) experienced similarly high tissue oxygenation during net photosynthesis. However, anemones maintained under bright light tend to have higher cytochrome *c* oxidase activity, which is in keeping with a lower photosynthetic oxygen production owing to their contraction during prolonged high irradiance (Shick, unpubl. data; see also Shick and Dykens, 1984).

An alternative interpretation of the data on cytochrome *c* oxidase is that the activity of this enzyme (and that of the mitochondrial respiratory chain in general) might be lower in zooxanthellate than in aposymbiotic specimens because mitochondria experiencing high oxygen levels have the potential for elevated production of superoxide radicals. Much of the superoxide production in cells occurs via autooxidation of the respiratory chain components NADH dehydrogenase and ubiquinone (Turrens and Boveris, 1980), and its production increases with P_{O_2} (Freeman and Crapo, 1981; Turrens *et al.*, 1982); thus, total mitochondrial production of superoxide radicals in a tissue is a function, both of the concentration of respiratory chain components, and of P_{O_2} (see also Shick and Dykens, 1985). Therefore, the high activity of cytochrome *c* oxidase in aposymbiotic *Aiptasia pulchella*, compared with its symbiotic clonemates, may reflect not simply a compensation for hypoxia in the former, but also an avoidance of oxidative stress in the latter.

The present study demonstrates that, depending on the size and morphology of the species being examined, respiration is variably enhanced by hyperoxia at a level that occurs in the illuminated tissues of zooxanthellate anthozoans, even under well-stirred, turbulent conditions that minimize the thickness of the diffusive boundary layer. Therefore, if the rate of respiration measured in the dark is to be taken as representative of that in the light, then dark respiration must, in some cases, be measured in hyperoxic water. It should be noted that the imposition of exogenous hyperoxia does not affect the thickness of the boundary layer; rather, it steepens the diffusion gradient across the boundary and thus enhances delivery of oxygen to the tissues. Such an enhancement of oxygen delivery simulates the effect of production of oxygen within the tissues, a condition that prevails when the symbiosis is illuminated. Whether and how much this enhances oxygen consumption depends on the size and morphology of the polyps, the hydrodynamic porosity of the colony, and the amount and perhaps the nature (laminar or turbulent) of water flow.

The elevation of respiration by hyperoxia, and the observation that the symbiosis produces more oxygen than it consumes, emphasize that, during daylight, the relevant source of oxygen for respiration is endogenous. Therefore, water movement and the thickness of the diffusive boundary layer around the polyps or colony may at that time be more relevant to the removal of endogenously produced oxygen, which, at high concentrations, inhibits photosynthesis in the zooxanthellae (Black *et al.*, 1976; Downton *et al.*, 1976) and necessitates greater defenses against oxidative stress in the host and its symbionts (Shick and Dykens, 1985; Lesser and Shick, 1989).

Elevation of respiration by hyperoxia *per se* occurs irrespective of its possible further enhancement by photosynthate translocated from photosynthesizing zooxanthellae (Edmunds and Davies, 1988). Although the provision of oxygen by the zooxanthellae to the host traditionally has been viewed as only supplementary, the current results suggest that the higher oxygen levels in the tissues of zooxanthellate cnidarians reduce the amount of respiratory apparatus (*e.g.*, cytochromes) that the host maintains. This saving may be somewhat offset by the need for higher levels of defenses against oxygen toxicity in zooxanthellate individuals.

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Ornithine Decarboxylase Exhibits Negative Thermal Modulation in the Sea Star *Asterias vulgaris:* Potential Regulatory Role During Temperature-Dependent Testicular Growth

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Abstract. The common northern sea star *Asterias vulgaris* is exposed to seasonal variation in temperature from −2 to 17°C. *A. vulgaris* exhibits an annual reproductive cycle, *i.e.*, the testes increase slowly in size during fall and winter, and reach maximal size in early spring. Slow testicular growth in the winter has been attributed to low field temperatures. Previous studies indicate that the specific activity of ornithine decarboxylase and the levels of the polyamines putrescine, spermidine, and spermine decrease in mid-winter and increase in the spring, coincident with changes in field temperatures. Kinetic studies show that ornithine decarboxylase assayed from individuals collected in March exhibits negative thermal modulation (K_m of ornithine is 0.22 m*M* and 0.65 m*M* at 15 and 0°C, respectively). Q_{10} values are highest at low substrate concentrations and at low temperatures. We hypothesize that during the cold winter months a decrease in the amount of ODC and an increase in the apparent K_m causes polyamine synthesis to decline, leading to decreased growth and development of the testis. We suggest that thermal modulation of ODC (and polyamine synthesis) is a mechanism by which seasonal temperature fluctuations influence seasonal spermatogenesis in *A. vulgaris*. We further suggest that growth of various tissues in many other ectothermal invertebrates may be similarly controlled.

Introduction

The biogenic polyamines spermidine and spermine and their diamine precursor putrescine are organic cations with multiple biological functions. Polyamines are known principally for their essential role in cell proliferation and for interactions with anionic molecules such as nucleic acids and membrane phospholipids in mammalian cell lines and tissues, both normal and neoplastic. However, the exact mechanisms by which polyamines influence cell proliferation are not fully understood.

Polyamine synthesis is controlled via the rate-limiting enzyme ornithine decarboxylase (ODC) (1). The activity of mammalian ODC varies in response to a wide variety of stimuli including growth factors, hormones, and drugs (2). The inducibility and short half life of ODC [<15 min, (1)] suggest that the activity of the enzyme (and thus polyamine synthesis) is highly regulated. The activity and kinetics of mammalian ODC have been characterized in numerous studies (reviewed by ref. 1). ODC has not been characterized for any invertebrate with the exception of a partial kinetic analysis of the enzyme in a snail (3).

The role of ODC and polyamines during cell proliferation in invertebrates should be similar to cell proliferation in mammals. However, relatively few studies have examined polyamine metabolism in the tissues of invertebrates. This is unfortunate because many invertebrates, particularly larvae and juveniles, show high seasonal and yearly growth rates. Polyamines are present in tissues of several invertebrates, although their distribution varies greatly among tissue types (4, 5, 6, 7, 8, 9, 10). In addition, polyamine levels increase significantly during periods of mitotic and meiotic cell proliferation in the testes of the sea star *Asterias vulgaris* (11). Cell proliferation in the testes, demonstrated by thymidine incorporation, also increases during exposure to extrinsically applied polyamines (12).

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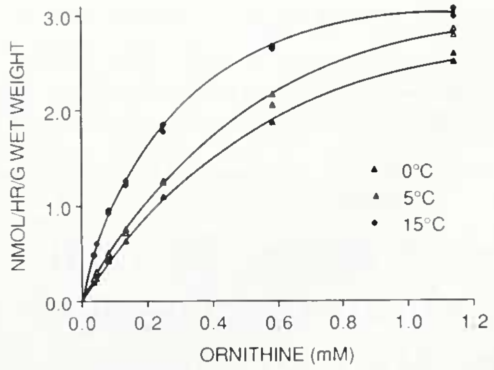

Figure 1. Activity of ornithine decarboxylase measured at various substrate (ornithine) concentrations and at assay temperatures of 0, 5, and 15°C. All assays were performed in duplicate.

Many invertebrates are exposed to daily or seasonal changes in temperature and demonstrate temperature-dependent growth characteristics. Although temperature is often cited as an important modulator of growth, the mechanisms by which temperature-dependent growth is regulated remain obscure. We hypothesize that temperature regulation of ornithine decarboxylase activity and polyamine synthesis may be one mechanism by which temperature regulates growth in ectothermal organisms. In this study we report the effect of assay temperature on the kinetic characteristics of ornithine decarboxylase extracted from the testes of *A. vulgaris*.

Materials and Methods

Adult specimens of *Asterias vulgaris* (8–10 cm arm length) were collected from a depth of 3 meters at the mouth of the Piscatequa River in Portsmouth, New Hampshire, in March 1987. Testes were removed from three individuals and pooled for ODC extraction and analysis.

Fresh testes were homogenized (20% w:v, 1:4) on ice with a glass Teflon homogenizing apparatus in a buffer containing 50 m*M* KH_2PO_4, pH 7.5, 0.2 m*M* EDTA, 5 m*M* dithiothreitol and 50 μM pyridoxal 5-phosphate. The crude extract was centrifuged for 30 min at 20,000 × *g* at 0°C. The supernatant was used for enzyme activity determinations.

The specific activity of ODC was determined by a procedure modified from Landy-Otsuka and Scheffler (13) and Smith (14). The specific activity of ODC was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C] ornithine hydrochloride (CFA.423, Amersham, 58 mCi/mmol). The enzyme reaction was performed in a 16 mm (ID) borosilicate test tube capped with a double-seal rubber stopper (Kontes, K-882310) penetrated by a plastic centerwell (Kontes K-882320). The centerwell contained a 2 × 3 cm square of Whatman #1 filter paper saturated with 100 μl NCS tissue solubilizer (Amersham). First, 150 μl of the above supernatant was added to each tube, and the reaction was initiated by adding 30 μl of 0.5 μCi DL-[1-^{14}C] ornithine hydrochloride and cold L-ornithine (Sigma, final concentration of total L-ornithine, approximately 1.2 m*M*). The reaction was stopped after 90 min at 15°C by injecting 0.5 ml 5% trichloroacetic acid into all tubes. These tubes stood for at least 1 h to permit maximum absorption of CO_2. Control tubes used to determine endogenous $^{14}CO_2$ release were prepared by adding first 0.5 ml TCA followed by 150 μl of ODC supernatant and 30 μl L-ornithine. The filter paper was removed and placed in scintillation vials containing 4.0 ml Beckmann NA scintillation fluid. The vials stood in the dark overnight so that the chemiluminescence would be reduced before the radioactivity was measured in an LKB Excel liquid scintillation counter.

The activity of ODC was measured at seven concentrations ranging from 0.0351 to 1.144 m*M* ornithine at assay temperatures of 0, 5, and 15°C. These temperatures are within the normal range of temperature to which the sea stars are exposed during the year (−2 to 17°C). All of the assays were performed in duplicate. Activities were expressed as nmoles ornithine converted to putrescine per h per g wet weight tissue. The apparent Michaelis constant (K_m) was determined from double reciprocal plots. Although potential problems may arise using crude supernatants to determine enzyme activity, relative differences in enzyme activities should reflect biological differences with respect to changes in temperature.

Results

The activity of ODC increased hyperbolically with an increase in substrate (ornithine) concentration (Fig. 1). The affinity of the enzyme for ornithine decreased with the assay temperature at all substrate concentrations

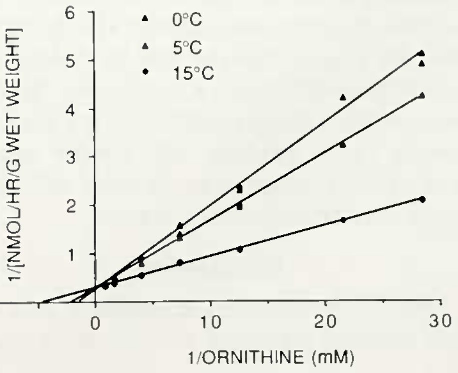

Figure 2. Double-reciprocal plot of the activity of ornithine decarboxylase *versus* substrate concentration. All assays were performed in duplicate.

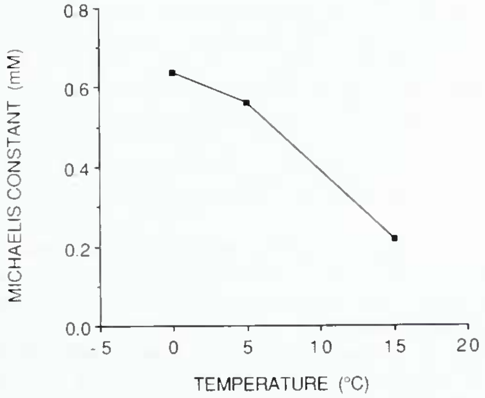

Figure 3. Relation of the apparent Michaelis contant (Km, mM), as determined from the double-reciprocal plot of the activity of ornithine decarboxylase, and assay temperature. A decrease in the assay temperature caused an increase in K_m and, therefore, a decrease in the apparent affinity of the enzyme for the substrate.

tested, indicating temperature-dependent enzyme-substrate affinity parameters.

The double reciprocal plots (Fig. 2) of ODC activity show that the assay temperature did not affect the maximal velocity of ornithine decarboxylase, as V_{max} did not change with temperature between 0 and 15°C. An increase in the slope of the line was apparent with a decrease in assay temperature, indicating an increase in the apparent K_m. A plot of apparent K_m *versus* temperature, illustrating negative thermal modulation (increased K_m with a decrease in temperature), is shown in Figure 3.

The Q_{10} values for the ODC activities were calculated between both 0 and 5°C and 5 and 15°C (Table I). Q_{10} values were significantly higher (sign test, $P < 0.05$) at the low temperatures at all substrate concentrations, ranging from 2.21 to 2.59 between 0 and 5°C. Q_{10} values were lowest at the high temperatures, ranging from 1.07 to 2.04 between 5 and 15°C. Q_{10} values of approximately unity indicated that ODC activity was essentially temperature-independent at high substrate concentrations (1.14 mM ornithine) between 5 and 15°C. These values show that ODC activity is temperature-dependent at low substrate concentrations and at low temperatures (regardless of substrate concentration), and temperature-independent at high substrate concentrations at higher temperatures only.

Discussion

The effects of temperature on organismal behavior, physiology, and the biochemistry of some cellular processes has been well documented for many ectothermic invertebrates. At the cellular level, the influence of temperature on enzyme activities is varied and dependent on the interactions of substrates and cofactors, translational and transcriptional control of enzyme production, and a host of other factors such as the metabolic pathway, type of tissue, organism, and the physical environment to which the organism is acclimatized or adapted (15).

Metabolic pathways influencing the rate of cell and tissue growth (including hyperplastic and hypertrophic growth) include those that influence the rate of energy production (glycolysis, Krebs cycle) and those that influence the rates of macromolecular biosynthesis (proteins, lipids, nucleic acids). Temperature acclimation and adaptation of many of the enzymes involved in energy production have been reviewed by Hazel and Prosser (16). Fewer studies have focused on temperature adaptation of those enzymatic reactions directly involved in macromolecule synthesis, particularly those involved in regulating macromolecule synthesis and function.

Polyamine biosynthesis, regulated by the activity of ODC, is directly involved in macromolecular synthesis and is considered to be one of the rate-limiting steps in the regulation of protein and nucleic acid metabolism, as well as cell growth (1, 2, 17, 18, 19). In this study we have investigated the effects of temperature on the kinetic characteristics of an invertebrate ODC obtained from the testes of the sea star *Asterias vulgaris*. *A. vulgaris* is seasonally exposed to temperatures ranging from −2 to 17°C (11). Watts *et al.* (11) found that significant decreases in testicular growth, polyamine levels (putrescine, spermidine, and spermine), and ODC activity were coincident with decreasing or low environmental temperatures. It was hypothesized that low field temperatures resulted in a decrease in polyamine biosynthesis by directly influencing the synthesis of ODC, either at the translational or transcriptional level.

In this experiment we have shown that ODC exhibits "negative thermal modulation" (15). Decreases in assay temperature resulted in increases in the apparent Km, thereby lowering the affinity of the enzyme for the substrate. Reduced enzyme affinity for the substrate, as well

TABLE I

Values of Q_{10} as determined from the activity of ornithine decarboxylase measured at various substrate concentrations

[ORNITHINE] (mM)	0–5°C	5–15°C
.0351	2.35	2.04
.0463	2.59	1.94
.0799	2.36	1.86
.1359	2.34	1.69
.2479	2.29	1.45
.5839	2.26	1.26
1.144	2.21	1.07

Values of Q_{10} were determined over the range of 0 to 5°C and 5 to 15°C.

as reduced ODC synthesis reported previously by Watts *et al.* (11) suggest that polyamine biosynthesis, which is necessary for cell and tissue growth, may be decreased during exposure to low temperature, thereby inhibiting growth.

The influence of temperature on the rate of ODC activity becomes more apparent when examined in terms of Q_{10}. The combined effects of reduced substrate binding and reduced kinetic energy available for enzyme activation at low temperatures produced higher Q_{10} values at apparent physiological substrate concentrations (*ca.* 0.2 m*M* ornithine; Watts, unpub.). In addition, Q_{10} values are highest when calculated at the reduced assay temperatures, causing temperature-dependent ODC activity and kinetics at low temperatures.

We hypothesize that the exposure of *Asterias vulgaris* to low temperatures decreases growth and development of the testis by negative modulation of polyamine synthesis. In individuals exposed to low temperatures, we suggest that polyamine synthesis may be negatively modulated by 1: a decrease in the amount of enzyme (either translational or transcriptional control), 2: an increase in the amount or binding of a proposed antizyme (1, 2), or 3: a decrease in the affinity of the enzyme for the substrate as indicated by changes in the K_m. Thermal modulation of polyamine metabolism may be a mechanism by which seasonal temperature fluctuations induce the seasonal patterns of growth observed in many ectothermal organisms. Further studies are needed to determine the extent to which temperature influences these processes in these organisms.

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CONTENTS

Annual Report of the Marine Biological Laboratory 1

INVITED REVIEW

Pardue, M. L., W. G. Bendena, M. E. Fini, J. C. Garbe, N. C. Hogan, and K. L. Traverse
Hsr-omega, a novel gene encoded by a *Drosophila* heat shock puff 77

BEHAVIOR

Buck, John
Unisex flash controls in dialog fireflies 87

DEVELOPMENT AND REPRODUCTION

Goldberg, Walter M., Ken R. Grange, George T. Taylor, and Alicia L. Zuniga
The structure of sweeper tentacles in the black coral *Antipathes fiordensis* 96

ECOLOGY AND EVOLUTION

Curtis, Lawrence A.
Parasitism and the movements of intertidal gastropod individuals 105
Lonsdale, Darcy J., and Sigrun H. Jonasdottir
Geographic variation in naupliar growth and survival in a harpacticoid copepod 113

GENERAL BIOLOGY

Pennington, J. Timothy, and Richard R. Strathmann
Consequences of the calcite skeletons of planktonic echinoderm larvae for orientation, swimming, and shape 121

PHYSIOLOGY

Dahlhoff, Elizabeth, Sabine Schneidemann, and George N. Somero
Pressure-temperature interactions on M_4-lactate dehydrogenases from hydrothermal vent fishes: evidence for adaptation to elevated temperatures by the zoarcid *Thermarces andersoni*, but not by the bythitid, *Bythites hollisi* 134
Michibata, Hitoshi, Hisayoshi Hirose, Kiyomi Sugiyama, Yukari Ookubo, and Kan Kanamori
Extraction of a vanadium-binding substance (vanadobin) from the blood cells of several ascidian species 140
Shick, J. Malcolm
Diffusion limitation and hyperoxic enhancement of oxygen consumption in zooxanthellate sea anemones, zoanthids, and corals 148

RESEARCH NOTE

Watts, Stephen A., J. Roy, and C. W. Walker
Ornithine decarboxylase exhibits negative thermal modulation in the sea star *Asterias vulgaris:* potential regulatory role during temperature-dependent testicular growth 159

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A Practical Guide to the Developmental Biology of Terrestrial-Breeding Frogs

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Abstract. Many frogs lay their eggs in water; the development of these frogs is well-known. However, many frogs reproduce on land; their eggs are large and have an altered early development. As examples, *Gastrotheca riobambae* broods its embryos in a pouch on the mother's back, and *Eleutherodactylus coqui* exhibits direct development with no tadpole stage. We provide practical information on obtaining eggs and embryos from these terrestrial-breeding species and on analyzing their development. Our aim is to make these species more accessible to researchers who are interested in the developmental and evolutionary consequences of terrestrial development.

Introduction

Our view of frog development is colored by the fact that most scientists work in the temperate climates of Europe, Asia, and North America. We expect frogs and other anuran amphibians to lay their eggs in water, to develop first into tadpoles, and to metamorphose later into adults. A number of anurans, particularly those found in the tropics, do not follow this life history (Lamotte and Lescure, 1977; Duellman and Trueb, 1986). Some anurans lack a free-living tadpole and develop directly to an adult morphology. Many anurans develop entirely on land and brood their embryos in such diverse places as the oviduct, a back pouch, the stomach, or the male's vocal sac. Given these terrestrial life histories, the name "amphibian" might not have been applied to this class had taxonomy begun as a tropical science, and our view of frog development might be totally different.

Anurans with terrestrial development generally have a small number (1–150) of very large eggs (3–10 mm), which differ in various aspects of development from typical anurans. Developmental biologists have noticed only a few of these animals including *Gastrotheca riobambae, Flectonotus pygmaeus,* and *Eleutherodactylus coqui. Gastrotheca riobambae* is an egg-brooding frog from Ecuador. The female incubates the eggs in a pouch on her back, and the young are born as advanced tadpoles. *Flectonotus pygmaeus,* from Venezuela, exhibits multinucleate oogenesis. The early oocytes have up to 2000 nuclei, of which all but one disappear (del Pino and Humphries, 1978). *Eleutherodactylus coqui,* from Puerto Rico, exhibits direct development. The large eggs are brooded on land by the male (Townsend *et al.,* 1984), and the froglets hatch after about three weeks.

Developmental studies to date have concentrated on oogenesis and early development in *G. riobambae* and *F. pygmaeus* (del Pino and Humphries, 1978; del Pino and Escobar, 1981; Elinson and del Pino, 1985; del Pino *et al.,* 1986; del Pino, 1989) and on organ formation and direct development in *E. coqui* (Lynn, 1942; Lynn and Peadon, 1955; Adamson *et al.,* 1960; Chibon, 1962; Elinson, 1990). Our discussion of these animals will reflect this bias, as we describe how to obtain and work with adults and embryos of *G. riobambae, F. pygmaeus,* and *E. coqui.*

Received 1 June 1990; accepted 27 July 1990.

Gastrotheca riobambae and Other Egg-Brooding Frogs (Hylidae)

Collection and maintenance[1]

Sites of collection. The majority of egg-brooding frogs inhabit the humid forests of northern South America, have very limited distribution ranges, and are rarely found (Table I). Species of *Gastrotheca* that inhabit the highlands of the Andes, however, occur in large numbers and are more easily collected. We will mostly discuss *G. riobambae,* the species with which we have extensive experience, and will make reference to *G. plumbea* and *F. pygmaeus,* which we have also maintained in captivity.

Gastrotheca riobambae occurs in the interandean valleys of northern Ecuador at altitudes of 2500–3200 m (Duellman and Hillis, 1987). Frogs are found along the banks of irrigation ditches and under stones in humid areas, as well as sitting on vegetation. This frog gives birth to tadpoles that develop in temporary pools and lakes. The free-living tadpole stage lasts several months. The large tadpoles of *G. riobambae* (around 70 mm total length) are the only amphibian larvae that exploit the standing bodies of water in the highlands of northern Ecuador.

Gastrotheca plumbea lives in cloud forests at elevations of 1300–2350 m on the Pacific slopes of the Andes in Ecuador (Duellman and Hillis, 1987). Both adults and young are found in axils of large bromeliads. *Gastrotheca plumbea* exhibits direct development, which means it lacks a free-living tadpole stage.

Flectonotus pygmaeus lives in axils of bromeliads, in the cloud forest at Estación Biológica de Rancho Grande, Maracay, Venezuela. This frog gives birth to advanced, non-feeding tadpoles that complete metamorphosis in about a week. The tadpoles are deposited in the water collected in axils of bromeliads (Duellman and Maness, 1980).

Table I

Geographic distribution of egg-brooding frogs

Genera of egg-brooding frogs	Approximate number of species[a]	Distribution[a]
WITHOUT POUCH		
Cryptobatrachus	3	Andes and Sierra Nevada de Santa Marta, Colombia
Hemiphractus	5	Panama, Pacific slopes of Colombia and northwestern Ecuador; upper Amazon Basin in Brazil, Ecuador, Perú and Bolivia[b]
Stefania	7	Highlands of the Guyana Shield in Venezuela and Guyana[c]
MARSUPIAL FROGS		
Flectonotus	2	Coastal Cordillera of northern Venezuela, Tobago and Trinidad[d]
Fritziana	3	Mountains of Southeastern Brazil (Guanabara, Minas Gerais, Rio de Janeiro, Sao Paulo)[d]
Gastrotheca	41	Panama, northern and western South America, southward to northern Argentina, east and southeastern Brazil

[a] Duellman (1977).
[b] Trueb (1974).
[c] Duellman and Hoogmoed (1984).
[d] Duellman and Gray (1983).

Terraria for laboratory maintenance. Our terraria for maintaining *Gastrotheca* and *Flectonotus* consist of wooden frames, 60–80 cm in length by 40 cm in width and 40–50 cm in height, with side walls of plastic mesh. The floor and the roof are made of wood. The roof has a large door, that fits tightly, and measures about half the length of the terrarium. Terraria of glass and of plastic are also used. In those cases, the cover is made of plastic or metal mesh to allow gas exchange.

The number of frogs per terrarium varies according to frog size. We keep about 8–10 adults of *G. riobambae,* 4–5 adults of the larger *G. plumbea,* or 12 *F. pygmaeus* per terrarium. *Gastrotheca riobambae* have been kept for up to five years, with reproduction occurring about once a year. *Gastrotheca plumbea* and *F. pygmaeus* have been kept for about two years, but their reproductive cycle and lifespan are not known.

Inside terraria we place large plastic trays with earth to cover the floor completely. Stones and hollow pieces of brick provide frogs with hiding places, and branches serve as perches. One or two small containers with water (about 5 cm in depth) are provided with stones to give support to the frogs. Each terrarium contains one or two small pots planted with *Tradescantia*. This plant grows easily and soon covers the terrarium. In addition, a bromeliad is planted, when available. Bromeliads with thorny leaves produce wounds in frogs and are not recommended. Terraria are placed near windows where there is ample sunlight and are kept moist by watering at least twice a week. Terraria are kept at 17–23°C. In native habitats of *G. riobambae*, the temperature fluctuates between 5°C at night to 23°C at midday.

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[1] South American wildlife is protected by law. Investigators interested in the collection of these frogs should check with the appropriate authorities in the country where they wish to work. Permission for the collection and export of frogs from Ecuador is given by the Director, Dirección de Desarrollo Forestal, Ministerio de Agricultura, Quito, Ecuador. A proposal, written in Spanish, should be sent several months in advance to the above address with a copy to the collaborating investigator or institution in Ecuador. It should be accompanied by the curriculum vitae of the investigator and a letter of support from the researcher's home institution. Duplicate collections must remain in Ecuador, usually at the Museo de Ciencias Naturales, Casa de la Cultura Ecuatoriana, Quito, Ecuador, or at the collection of one of the universities. To obtain permits, visiting scientists are required to give lectures about their work at the Museo de Ciencias Naturales or at the collaborating institution. Upon completion of the work, a report and copies of published works should be sent to Dirección de Desarrollo Forestal and to the collaborating institutions. Upon arrival in Quito, investigators are advised to visit the Dirección de Desarrollo Forestal as soon as possible to get their permits.

Investigators interested in *F. pygmaeus* are advised to check with the Estación Biológica de Rancho Grande, Maracay, Venezuela. A permit for collection is required from the Estación Biológica de Rancho Grande, and permits for export are obtained from the Oficina de Fauna, Ministerio de Agricultura y Cria, Caracas, Venezuela.

Favorite hiding places for both *G. riobambae* and *G. plumbea* are the cavities under stones and bricks and in axils of bromeliads. In addition, they hide in the crevices between plastic trays and the terrarium walls as well as under the vegetation. *Flectonotus pygmaeus* perches in axils of bromeliads and on the vegetation. Frogs bask in the sun and may remain in the same place for several days. Frogs are in the water often, so water must be changed frequently.

We have not tried terraria of larger height. However, Zimmermann (1983) described taller terraria with several vegetation levels and high humidity for the maintenance of tropical frogs. Those terraria have a frontal glass door and might be useful for maintaining large arboreal species of egg-brooding frogs.

Feeding. Adult *G. riobambae* are fed two to three times a week. The easiest, most efficient food consists of sowbugs (*Porcellio* sp.) or meal-worm larvae (*Tenebrio mollitor*), mixed with small pieces of dry dog food. Food is placed in shallow plastic containers (1.5–2 cm in depth) and is always given in the same place. Meal worms remain in feeding containers and in this way, frogs learn to eat dry food when capturing prey.

We have maintained *G. riobambae* successfully on only a sowbug diet; however, sowbugs become scarce in dry weather. To avoid food scarcity, feeding alternately with sowbugs or meal-worms and dry dog food gives excellent results. Dog food provides the frogs with carbohydrates, lipids, vitamins, and minerals. Outside the feeding containers, frogs ingest dirt from the terrarium floor with their prey and may obtain needed trace elements; frogs raised without dirt become weak. This diet is also well accepted by *G. plumbea*. *Flectonotus pygmaeus* is fed once or twice a day on large *Drosophila* caught from the wild. Small sowbugs and meal-worm larvae were not tried, but could probably be given successfully to these frogs.

Newly metamorphosed *G. riobambae* readily accept small meal-worm larvae, and this type of food is a key to successfully raising these frogs when used in combination with other small prey items. We mix larvae with dry dog food in very shallow plastic containers. Newly born *G. plumbea* have been raised successfully on the same diet given to young *G. riobambae*. In addition, young *G. plumbea* were fed large *Drosophila* caught from the wild. Juvenile *G. riobambae* and *G. plumbea* reach the adult stage 8–12 months after metamorphosis. Newly metamorphosed *F. pygmaeus* are quite small, and we have had no success in raising them. Very small meal-worm larvae, which are readily accepted by other species, were not tried on *F. pygmaeus*.

Amplexus and birth. Before amplexus, *G. riobambae* males call frequently. Amplexus occurs on land and it lasts for 24–48 h before egg-laying begins. Egg laying lasts about 6–8 h. During egg laying, the male introduces his feet inside the female's pouch. As each egg leaves the female's cloaca, the male catches it with his heels and toes and moves it inside the pouch, the opening of which is about a centimeter anterior. In this way, eggs do not touch the ground. Eggs leave the female's cloaca, one at a time, at intervals of 30–60 s. A few eggs are lost and remain in the soil at the end of amplexus. Fertilization probably occurs during the egg's journey from the female's cloaca to the pouch.

After amplexus, the female places herself tight against the cavity of a stone or other object, probably to help in pouch distension and in the arrangement of embryos in one or two even layers (del Pino *et al.*, 1975). Incubation of embryos lasts a mean of 100 days, during which time the wet weight of embryos increases three-fold, while the dry weight remains constant (del Pino and Escobar, 1981). At birth, the total weight of embryos equals one third to one half the weight of the female. Females become so swollen with embryos that their movements are greatly reduced.

At birth, the female enters the water, introduces the long toes of her hind legs inside the pouch, and aids in the removal of tadpoles. She supports herself with her front legs against the walls of the water container or against a stone. Tadpoles at birth measure 18–20 mm in total length (del Pino *et al.*, 1975).

In captivity, amplexus of *G. riobambae* occurs between September and February, the period with heavier rainfall in Quito. Sometimes frogs mate in June. These periods seem to coincide with times when most reproduction occurs in nature; however, females with embryos can be collected throughout the year. By administering human Chorionic Gonadotropin (hCG) to male and female frogs, described later, amplexus and reproduction can be obtained in captivity at any time of the year.

Amplexus in *G. plumbea* also occurs on land, but details have not been documented. Incubation lasts about 120 days (del Pino and Escobar, 1981). At birth, the mother actively aids in the elimination of offspring by stretching the opening of the pouch and digging young out of the pouch with her hind feet, as in *G. riobambae* (Duellman and Maness, 1980). Brooding females have been found in June and July, and frogs in captivity gave birth in September (Duellman and Maness, 1980). Amplexus and birth for *F. pygmaeus* have been described by Duellman and Maness (1980). Amplexus occurs on land, and at birth the female deposits larvae in water. Embryos were incubated for 29 days in captivity (del Pino and Escobar, 1981). The breeding season of *F. pygmaeus* at Estación Biológica de Rancho Grande spans from April until early November, the time of the year with the heaviest rainfall (Duellman and Maness, 1980). We have not tried hormonal stimulation of reproduction in *G. plumbea* or *F. pygmaeus*, but both species have mated spontaneously in the laboratory.

Care of tadpoles. The extensive use of pesticides and urban growth have diminished the populations of *G. riobambae*, so frogs must be collected from remote localities. To ensure a supply of frogs, we have developed methods for raising frogs from tadpoles.

Tadpoles are best maintained in large tanks with at least one cubic meter of water, a depth of 30–50 cm, and a temperature of 17–21°C, without changes of water. Tanks are provided with stones as hiding places, a large plant (*Cyperaceae*) whose roots, floating stems, and leaves provided places for tadpoles to sit near the water surface, and a few branches of *Elodea*. These tanks support an abundant population of algae and protozoans that become the main constituent of the tadpole diet. About 70–100 tadpoles are raised per tank. Little or no cannibalism is observed, and tadpoles grow normally, even when newly born tadpoles are placed with older ones.

Tadpoles of *G. riobambae* are voracious eaters. The easiest, most effective diet has been small pieces of dry dog food or rabbit food given once or twice a week, supplemented by the algal growth in the tanks. Care should be taken not to contaminate the water with too much food. Tadpoles reached 70 mm total length before metamorphosis, at about 3 months after birth. Frogs measured 18–25 mm snout-vent length, looked healthy, and survived after metamorphosis.

Tadpoles have also been kept in small glass and plastic aquaria of 10–20 liters capacity. Without a stable population of algae, the water becomes contaminated and requires weekly changes. In some instances, aquaria were aerated by means of an aquarium pump, but we found no advantage in aeration. Tadpoles of *G. riobambae* have well-developed lungs by the time of birth and take oxygen from air. Population densities in aquaria correspond to 1–3 tadpoles per liter of water. Higher densities result in cannibalism and early metamorphosis. Under crowded conditions small tadpoles, 50–60 mm total length, reached metamorphosis in only 40 days at 18°C. The newly metamorphosed frogs were small (10–15 mm snout-vent length), extremely weak, and often died at metamorphosis or soon thereafter.

We have tested a variety of other diets for tadpoles. Diets included cooked and raw meat; cooked lettuce, chard and spinach; chicken feed hardened with agar; egg yolk from cooked eggs, and fish food for aquaria (Tetramin, Tetra Werke, 4520 Melle, Federal Republic of Germany). Of these diets, fish food is the best, followed by chicken feed hardened with agar.

Flectonotus pygmaeus produces about six advanced tadpoles per breeding season. In the laboratory, tadpoles have been kept in shallow water containers. These tadpoles do not eat and only need 2–4 weeks of aquatic living to reach metamorphosis (Duellman and Maness, 1980).

Maintenance at lower altitudes. The habitat of *G. riobambae* is the high montane environments in northern Ecuador (2500–3200 m altitude). Atmospheric pressure, oxygen availability, length of daylight, and amount of sunlight as well as temperature of the *G. riobambae* habitat differ from the conditions of most laboratories where studies on development are conducted. Change in altitude and temperature often result in frog death. We had the experience of raising *G. riobambae* at the aquaria of the German Cancer Research Center in Heidelberg, Federal Republic of Germany. Most female frogs that were incubating embryos died a few days after arrival, but one frog gave birth successfully and provided us with tadpoles, from which we obtained a supply of frogs.

Tadpoles were raised in small glass aquaria at a density of three tadpoles per liter. Aeration was provided with aquarium pumps. Tadpoles were fed fish food (Tetramin) and cooked lettuce and attained the normal length of about 70 mm before metamorphosis, about one month after birth. Newly metamorphosed frogs ate flies and small crickets readily. Eight months after birth, frogs reached adult size and began to sing. Adult frogs were maintained in terraria as previously described. Temperature was adjusted to 17–18°C and the light regime was a 12-h light/dark cycle. Frogs were fed adult house-flies and medium-size crickets, which were often dusted in powdered vitamins and minerals, Osspulvit (Zimmermann, 1983), to supplement the diet. Amplexus was observed on several occasions; however, egg laying did not occur.

Accelerated development has been reported previously at lower altitude. In Holland, Hoogmoed (1967) obtained metamorphosis of *G. riobambae* tadpoles in 41 days after birth at a temperature of 21–26°C. In contrast, tadpoles normally require several months of aquatic living to reach metamorphosis in Quito. Water temperature as well as the change in altitude should be factors involved in the differences observed.

We induced amplexus and reproduction by administering hCG intraperitoneally to both male and female frogs, as described later. Amplexus and fertilization occurred normally. Eggs began to cleave, but died at around the time of gastrulation. Lower altitude seems to affect the physiological condition of frogs and may affect the quality of eggs. For instance, the induction of oocyte maturation by progesterone *in vitro*, took 10 h longer in Santiago de Chile than in Quito (de Albuja *et al.*, 1983). Auber-Thomay and Letellier (1986) obtained regular and spontaneous reproduction of *G. riobambae* in France. Incubation took 41–74 days, but half of the eggs from 10 frogs failed to develop. In contrast, 75–108 days of incubation are needed in Quito, but almost all of the eggs (99.4%) developed (del Pino and Escobar, 1981). Our experience, and that of others, indicate that successful reproduction, as it happens in Quito, is rare at lower altitudes.

Oogenesis, fertilization, and the culture of embryos

Analysis of oogenesis. Egg-brooding frogs have synchronous oogenesis, which means that only one batch of oocytes grows in the ovary at one time (del Pino *et al.*, 1986). Full-grown oocytes are the largest documented among anurans, reaching 2.5–10 mm in diameter, depending on the species (Table II). Their volume is primarily due to yolk, and oocytes of *G. riobambae* actually have less rRNA than oocytes of *Xenopus laevis*, which are $^1/_{16}$th the volume (del Pino *et al.*, 1986).

In some species of egg-brooding frogs, oocytes contain many nuclei (4–2000 nuclei depending on species) during the previtellogenic period (Table II; del Pino and Humphries, 1978). At vitellogenesis, only one nucleus remains as the oocyte's germinal vesicle while the rest degenerate. This type of oogenesis is called multinucleate oogenesis (del Pino and Humphries, 1978), and unfortunately, the analysis of it is limited by the availability of frogs. Marsupial frogs with multinucleate oocytes are mostly large frogs (Table II) that live in cloud and humid forests and are rarely found. The most accessible species encountered so far is *F. pygmaeus*.

To study oogenesis, ovarian pieces are removed from the body cavity of the frog or tadpole and are placed in modified Barth Solution (MBS) (Gurdon, 1968), amphibian Ringer's, or in other amphibian saline solutions. MBS contains 88 m*M* NaCl, 1 m*M* KCl, 2.4 m*M* $NaHCO_3$, 0.82 m*M* $MgSO_4$, 0.33 m*M* $Ca(NO_3)_2$, 0.41 m*M* $CaCl_2$, 10 mg/l each benzylpenicillin and streptomycin sulphate and 2 m*M* Tris-HCl (or 10 m*M* Hepes), pH 7.6.

Methods for the cytological observation of germinal vesicles in mono- or multinucleate oocytes of egg-brooding frogs do not differ from those used with other amphibia (Macgregor and del Pino, 1982). The large amount of yolk stored in large oocytes, however, is bothersome for cytology as well as for the extraction of nucleic acids.

Fixation of oocytes for cytological studies needs to be modified due to the yolk content of oocytes. It is important to increase the volume of fixative and the length of fixation times. Smith's fluid (Rugh, 1965) is our fixative of choice for paraffin and plastic sections. One or two yolky oocytes of *G. riobambae* are fixed in 10–20 ml of Smith's fluid for 12–24 h in the dark, washed in distilled water with several changes for 24 h, and stored in 4% neutralized formalin until processing. Yolky oocytes fixed in Smith's are particularly easy to section, when embedded in paraffin. Material embedded in plastic resins, like JB-4 (Polysciences), provide better resolution than paraffin sections.

Small oocytes, taken from large tadpoles and newly metamorphosed frogs, can be processed for electron microscopy according to standard methods. Large, yolky oocytes should be cut into smaller pieces during glutaraldehyde fixation to allow better penetration of fixative. Thick epon sections (0.5–1 μm thickness) of oocytes, fixed in glutaraldehyde and postfixed in osmium tetroxide, can be used for light microscopy. Sections are placed on a drop of water over a clean slide and, after drying, are mounted with immersion oil for light microscopy (del Pino *et al.*, 1986).

The study of nucleic acids in large oocytes of *G. riobambae* is affected by the amount of yolk. The volume of working solutions needs to be increased 10 times in comparison to *X. laevis* oocytes due to the large oocyte volume. Nucleic acids cannot be totally cleaned from yolk contaminants without losing small RNA molecules (del Pino *et al.*, 1986).

The large size of oocytes in egg-brooding frogs is, at first, attractive for microinjection experiments. There are, however, several limitations. The small number of eggs and oocytes per female (Table II) prevent their extensive use. Eggs are uniformly pale, and the animal pole cannot be easily distinguished, a limitation when microinjection into the germinal vesicle is desired. Large egg size is due to yolk content, and the germinal vesicle of *G. riobambae* oocytes is equivalent in size to the germinal vesicle of *X. laevis* oocytes (del Pino *et al.*, 1986). Microinjection into oocytes and eggs of *G. riobambae* is further complicated by their high internal pressure. Oocytes and eggs often burst after microinjection, although this can be prevented by injecting oocytes kept in a humid chamber or in 5% Ficoll in an amphibian saline. The easily available oocytes of *X. laevis* provide superior material for microinjection experiments.

Hormonal induction of oocyte maturation, ovulation, and mating in G. riobambae. Large oocytes within the ovarian follicle can be induced to undergo germinal vesicle breakdown (GVBD) *in vitro* by exposure to hCG or progesterone. In contrast, oocytes denuded from the follicular wall undergo GVBD only in response to progesterone. Treatment of ovarian follicles with hCG results not only

Table II

Oogenesis and development of egg-brooding frogs

Frog species[a]	Snout-vent length of female (mm)	Number of oocyte nuclei 1 = mononucleate 2 = multinucleate	Egg diameter (mm)	Clutch size	Development 1 = tadpole 2 = direct
FROGS WITHOUT POUCH					
Cryptobatrachus boulengeri	65	—	4	26	2
C. fuhrmanni	60	1	4	28	2
Hemiphractus bubalus	58[c]	1	—	—	2
H. fasciatus	57[c]	1	7	13	2
H. johnsoni	68[c]	2	9	18	2
H. proboscideus	56[c]	1	—	—	2
H. scutatus	70[c]	1	10	10	2
Stefania evansi	64[d]	2	9[d]	11[d]	2
S. ginesi	61[d]	—	9[d]	8[d]	2
S. goini	93[d]	—	9[d]	15	2
S. woodleyi	61[d]	—	9[d]	6[d]	—
MARSUPIAL FROGS					
Frogs with pouch type one[b]					
Flectonotus fitzgeraldi	19[e]	2	3	3	2
F. pygmaeus	26[e]	2	3	7	2
Fritziana fissilis	26[e]	1	3	10[e]	1
F. goeldii	32[e]	1	4	15[e]	1
F. ohausi	25[e]	1	—	—	1
Frogs with pouch type two[b]					
Gastrotheca guentheri	82	2	—	—	2
G. andaquiensis	74	—	9	10	2
G. cornuta	74	2	10	10	2
G. dendronastes[f]	69	2	8	12	2
G. longipes	95	—	8	17	2
G. ovifera	81	2	8	32	2
G. microdisca[g]	75[g]	2	8	23[g]	2
G. walkeri[h]	60	2	7	19[h]	2
G. weinlandii	85	2	10	14	2
Frogs with pouch types three and four[b]					
G. christiani[i]	36	1	4	10	2
G. excubitor	41	1	6	10	2
G. galeata[j]	52[j]	1	5	20	2
G. griswoldi	41	2	8	12	2
G. ochoai	38	1	5	—	2
G. testudinea	60	1	5	30	2
Frogs with pouch types five and six[b]					
G. argenteovirens	42	1	3	35	1
G. aureomaculata	75	—	5	70	1
G. fissipes	81[g]	2	10[g]	24	2
G. gracilis	40	1	4	68[i]	1
G. lojana	60	1	3	—	1
G. nicefori	73	—	8	30	2
G. peruana	53	1	3	—	1
G. plumbea	69	1	5	28	2
G. orophylax[k]	59[k]	1	6[k]	21[k]	2
G. marsupiata	42	1	2.5	138	1
G. monticola	59[l]	1	3	80	1
G. riobambae	49[l]	1	3	128	1

[a] Duellman (1977).
[b] del Pino (1980).
[c] Trueb (1974).
[d] Duellman and Hoogmoed (1984).
[e] Duellman and Gray (1983).
[f] Duellman (1983).
[g] Duellman (1984).
[h] Duellman (1980).
[i] Laurent et al. (1986).
[j] Trueb and Duellman (1978).
[k] Duellman and Pyles (1980).
[l] Duellman and Hillis (1987).

in GVBD but also in ovulation (de Albuja *et al.*, 1983). The responses of *G. riobambae* oocytes to these hormones are equivalent to *X. laevis* and suggest that there are similar mechanisms for the conversion of an oocyte into an egg. The time of the response is longer in *G. riobambae*. The large oocytes of *G. riobambae* need an exposure of 15–24 h to progesterone to undergo GVBD compared to 6–8 h for *X. laevis* oocytes (de Albuja *et al.*, 1983).

To induce ovulation, female *G. riobambae* are injected intraperitoneally with hCG. The amount given ranges from 200 to 1000 IU of hCG, administered in one or two doses at 6–24 h intervals. Ovulation and egg release occurred 29–76 h after hormone injection (de Albuja *et al.*, 1983). When a female injected with hCG is placed in a terrarium with male frogs, amplexus and mating occur readily. In some instances, the male was simultaneously stimulated by the intraperitoneal injection of 200–500 IU of hCG. Hormonal stimulation of amplexus and mating in *G. riobambae* greatly facilitates the study of reproduction in this frog.

The maternal pouch as an indicator of the female's reproductive condition. The pouch of *G. riobambae* is a sac of integument located on the back of the female, under the dorsal skin. The pouch is essentially independent from the skin, except at the aperture, where it is continuous with the dorsal skin. Pouch integument of frogs that are not incubating embryos is similar to frog skin. It differs from skin by the presence of abundant mucous glands, fewer serous glands, and the lack of keratinization of the epithelial layer (Jones *et al.*, 1973; del Pino *et al.*, 1975).

The pouch of *G. riobambae* opens in the midline of the female's back and the aperture has an inverted V or U shape. The pouch can be open or closed according to the reproductive condition of the female (Fig. 1). The pouch of females with small ovaries is open; it closes when ovaries are large and the female is ready for reproduction, and it remains closed during the period of embryonic incubation. The pouch opens again at birth (del Pino, 1983). The condition of the pouch aperture provides an important parameter for the selection of female frogs that can reproduce readily. The dose of hCG needed to stimulate amplexus and mating in frogs with a closed pouch is smaller than that in frogs with open pouches. Similarly, oocytes of frogs with closed pouches require shorter periods of exposure to progesterone to undergo GVBD (de Albuja *et al.*, 1983; del Pino, 1983).

Incubation of embryos results in great distension and attenuation of the pouch in *G. riobambae*. The pouch, which originally occupied a small portion of the female's back, increases in size during incubation until it occupies the entire back and sides of the body. During the first weeks of incubation, the walls of the pouch become highly vascularized and grow between embryos, forming partitions that tightly envelop each embryo in a vascularized chamber. Embryos, in turn, develop disk-shaped gills, the bell gills (Noble, 1927), that envelop the growing embryo in a vascularized gill sac.

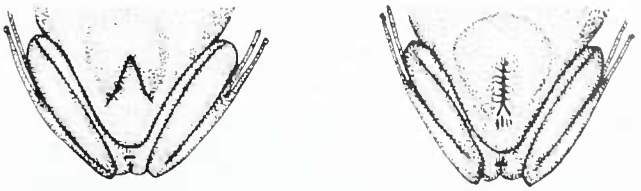

Figure 1. The pouch on the back of a *Gastrotheca riobambae* female. a. When the female is not incubating embryos, the pouch has an inverted V-shaped opening with smooth borders. b. When the female is ready to ovulate or is incubating embryos, the pouch closes with the borders touching except at the posterior end. (From del Pino, 1983.)

Fertilization. Fertilization in *G. riobambae*, *G. plumbea*, and *F. pygmaeus* is external, but it does not occur in water as in most frogs. Eggs are fertilized during the journey from the female's cloaca to the pouch, as already discussed. Since the requirements for *in vitro* fertilization are unknown, we depend on normal mating of frogs to obtain fertilized eggs.

When testis of *G. riobambae* are macerated in full strength MBS, sperm cells show little motility, but remain alive. Saline solutions of low ionic strength (like 10% MBS) do not trigger sperm motility. Such media, in fact, result in rapid swelling and bursting of sperm. *Xenopus laevis* sperm, in contrast, swim actively in solutions of low ionic strength before dying (Wolf and Hedrick, 1971). The experience of mixing both gametes for *in vitro* fertilization has, so far, been unsuccessful. The length of time needed for the process and the possible requirement of pouch secretions to activate sperm are factors that need to be considered for an *in vitro* fertilization method.

Culture of embryos. Embryos of *G. riobambae* can be removed from the pouch without affecting the mother or the development of other embryos in the pouch. Embryos are removed with a wide probe or a blunt pair of forceps. With frequent removal of embryos and handling of the frog, some embryos die and desiccate in the pouch. Dead embryos do not affect the development of the brood. Gentle handling of the frog with a piece of wet cloth helps to avoid desiccation, and many embryos survive in the pouch. In *F. pygmaeus*, the borders of the pouch are firmly sealed. Removal of embryos breaks the seal, but the remaining embryos will continue to develop.

During early stages of incubation, removal of embryos is easy. Newly fertilized eggs can be obtained by slight pressure on the female's back in *G. riobambae*. Later, with the development of embryonic chambers, pouch tissue adheres firmly to the jelly capsule of each embryo, and the capsule and bell gills can be ruptured during removal of embryos. Dipping the tip of the probe in MBS, before insertion into the pouch, helps in the clean separation of pouch tissue from embryos.

Segmenting eggs and early embryos of *G. riobambae* die in saline solutions of low ionic strength (*e.g.*, 10% MBS), but survive in full strength MBS or other amphibian salines. Advanced embryos (del Pino/Escobar stages 20–25) survive in solutions of high and low ionic strength (del Pino *et al.*, 1975). The tolerance of advanced embryos to low salt concentration is a preparation for the free-living tadpole stages.

For *in vitro* culture, segmenting eggs and early embryos are placed in 5% Ficoll in an amphibian saline. Embryos at the onset of cleavage have been cultured for 20 days in a tissue culture well filled with the above solution. Ficoll prevents swelling, which occurs in saline solutions and which inhibits gastrulation and leads to bursting. Culturing in Ficoll permits observations of blastula and gastrula stages. Successful culture of cleavage stage embryos was also accomplished by placing embryos in MBS after removing them from the pouch and transferring them to 2-ml wells of disposable tissue culture plates with a 10 μl drop of MBS. The amount of liquid around the embryo should be very small. The well is then filled with mineral oil. Once or twice a week, embryos are washed in a large dish with MBS to retard fungal growth, although it is easy to damage embryos during manipulation. Embryos can be cultured in this way for 2 to 4 weeks. We recommend using Ficoll in amphibian saline and incubation in 1–1.5 ml without changing the medium.

Advanced embryos (del Pino/Escobar stages 20–25) hatch from the jelly capsule quite easily. For culture, embryos are placed in a petri dish with full strength MBS, 10% MBS or tap water. The jelly capsule breaks, bell gills are resorbed into the peribranchial cavity, and embryos begin to swim as tadpoles. Embryonic growth in culture always becomes accelerated compared to development in the pouch. Some embryos develop edema when cultured at low salt concentrations.

Less efficient methods for the culture of segmenting eggs and early embryos of *G. riobambae* are the culture of embryos in a petri dish filled with MBS and the culture of embryos in humid chambers. In both cases, embryos hatch from the jelly capsule in a matter of days, and they swell and die. Alterations of the developmental pattern do occur; in particular, development of blood is deficient (del Pino *et al.*, 1975). Embryos (del Pino/Escobar stages 10–25) tolerate solutions of high ionic strength like 1.5 MBS and 2.0 MBS. However, the jelly capsule bursts and embryos swell and die in about 10 days. Development is retarded under those conditions.

Normal development

Development of *G. riobambae* follows the amphibian pattern, with several striking differences. Development is very slow, requiring about 12 h for first cleavage, a week until gastrulation begins, and another week for the completion of gastrulation (Elinson and del Pino, 1985). Gastrulation results in the formation of a small group of cells, called the embryonic disc, from which the embryo's body arises (del Pino and Elinson, 1983). The heart forms anterior to the head, and large bell gills develop, which envelop the embryo completely (del Pino and Escobar, 1981). Embryos up to del Pino/Escobar stage 17 are unpigmented and translucent, so that organ formation can be easily followed.

A staging table for the period of incubation has been prepared by del Pino and Escobar (1981), with further details of cleavage and gastrulation in Elinson and del Pino (1985). Salient features will be mentioned here (Fig. 2). Cleavage results in small animal micromeres and large vegetal macromeres (stage 5), and the blastula has a translucent roof (stage 6). The blastopore of gastrulation is hard

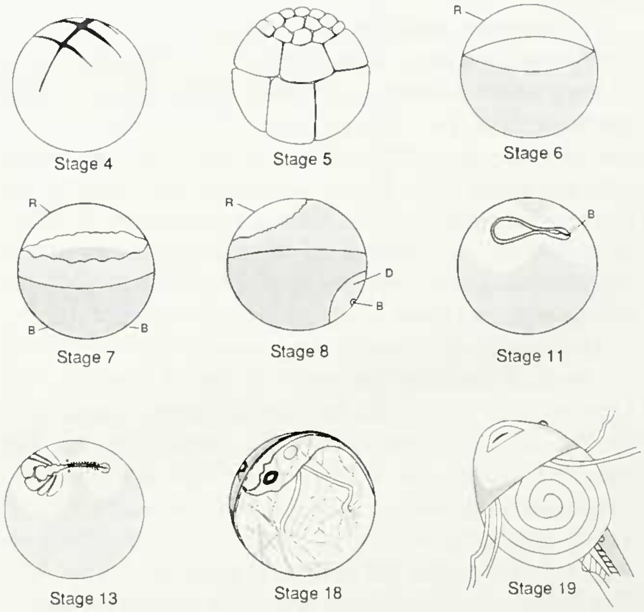

Figure 2. Selected del Pino/Escobar stages of *Gastrotheca riobambae* development. The first three cleavage furrows are vertical and cut slowly through the egg from animal to vegetal (Stage 4). The small, animal micromeres (Stage 5) become the translucent blastocoel roof (R) and the vegetal macromeres remain large (Stage 6). The faint blastoporal lip (B) forms near the vegetal pole at the start of gastrulation (Stage 7). The lip is usually impossible to see on living embryos, so the internal movement of cells along the blastocoel roof is a sign of gastrulation. The blastopore closes, and the lips become a discrete disc (D) of small cells (Stage 8). As the archenteron expands, the embryonic disc stretches to form the archenteric roof, and the whole embryo rotates with respect to gravity. Neural folds develop from the former disc cells (Stage 11). The embryo develops a central nervous system, branchial arches, and somites, all from the disc cells (Stage 13). The branchial arches produce bell gills, which expand to surround the embryo with vascular sheets (Stage 18). When the bell gills are dissected away, the characteristic tadpole is revealed (Stage 19). (Modified from del Pino and Escobar, 1981; Elinson and del Pino, 1985.)

to see because the eggs are white, but the migration of cells along the blastocoel roof is a sign of gastrulation (stage 7). The cells of the closing blastoporal lips form the embryonic disc (stage 8), which expands with the expansion of the archenteron. The embryo rotates so that the former disc is uppermost, and neural folds form from the disc cells (stage 11). The embryo develops as flat sheets of tissue with the central nervous system, somites, and branchial arches clearly visible (stage 13). The bell gills develop from the branchial arches and envelop the embryo (stage 18). With the absorption of yolk, the embryo acquires the typical tadpole appearance (stage 19). After birth, tadpoles can be staged using Gosner's (1960) generalized staging tables for aquatic species. Staging tables have not been prepared for egg-brooding frogs with direct development (Table II). These frogs also develop bell gills; there is partial development of the operculum, and precocious development of the limbs (del Pino and Escobar, 1981).

Manipulation of embryos

Few attempts have been made to manipulate embryos of *G. riobambae* for experimental purposes. Eggs of *G. riobambae* are uniformly pale, so that cleavage and gastrulation are hard to see in living embryos. By impregnating entire embryos with silver, the outline of cells can be seen (Kageyama, 1980). An embryo is removed from the pouch and its jelly coats are removed with forceps or by rolling the embryo on a piece of moist paper towel. The presence of air bubbles on the jelly should be avoided as they interfere with staining. The embryo is immersed, with agitation, in Stockerd's fixative (5 parts formalin, 4 parts acetic acid, 6 parts glycerol, 85 parts distilled water) for 10–20 s, and transferred immediately into a bath of distilled water. Longer fixation periods in Stockerd's fixative result in the rupture of cells. The egg is then placed in a dilute solution of silver nitrate prepared by adding one or two drops of a 0.5% stock solution of silver nitrate to 2 ml distilled water. Higher concentrations of silver nitrate produce dark staining of the egg. The egg is left in this solution for 1–2 min. It is washed in distilled water and exposed in distilled water, over a white background, to sunlight for a few minutes or until dark color develops. In the absence of sunlight, embryos can be exposed to the light of a strong lamp. Stained eggs are stored in 4% neutralized formalin.

Cell movement during cleavage and gastrulation can be followed by vital staining. Pieces of agar containing Nile blue sulphate are prepared by dissolving the stain in agar with boiling. The liquid is dried on a glass plate, and the resulting film is cut into small pieces. An embryo of *G. riobambae* is taken from the pouch, and its outer jelly removed by rolling the embryo on a piece of moist paper towel. The embryo is placed in a humid chamber, and spots of stain are applied to its surface using a piece of agar like a brush. After a few minutes, the embryo's surface is stained, and the excess stain, left on the jelly, is removed. Stained embryos are cultured under vaseline oil, and the changes in shape of the stain marks can be followed.

Segmenting eggs and embryos can be sectioned serially after fixation in Smith's fixative, as explained previously. Body formation and development of bell gills can be followed by means of whole mount permanent preparations. Embryos (del Pino/Escobar stages 11–17) are cut from the yolk and fixed in Bouin's solution. Embryos are then stained with borax-carmine and are mounted on a slide.

***Eleutherodactylus coqui* (Leptodactylidae)**

Collection and maintenance[2]

Sites and methods of collection. The genus *Eleutherodactylus*, with more than 400 species, is found from Mexico to northern Argentina and southeastern Brazil, as well as the West Indies and Florida. Most species have direct development of terrestrial eggs, but *E. jasperi* is viviparous (Drewry and Jones, 1976; Wake, 1978).

Eleutherodactylus coqui is the most common of sixteen congeners native to Puerto Rico (Rivero, 1978). The species is not available through any biological supplier (currently, no *Eleutherodactylus* species is), but must be captured in its native habitat.

As implied above, *E. coqui* can be confused with several other congeners that may occur sympatrically in various parts of the island. At least two of these species have threatened or endangered status, so it is important to contact a local herpetologist who is familiar with *E. coqui*.

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[2] Collection of any Puerto Rican species of frogs or eggs requires a permit. Permits can be obtained by writing to Department of Natural Resources, Apartado 5887, Puerta de Tierra, Puerto Rico 00906.

We urge any biologists interested in collecting frogs in Puerto Rico for laboratory investigations to contact a faculty member or research biologist at any of the following institutions to learn the most appropriate places for collecting without endangering local populations or disrupting ongoing field studies. Contacts include: Department of Biology, University of Puerto Rico, Rio Piedras, Puerto Rico; Department of Biology, University of Puerto Rico, Mayaguez, Puerto Rico; and Terrestrial Ecology, El Verde Field Station, GPO Box 3682, San Juan, Puerto Rico 00936.

The El Verde Field Station is located within the Luquillo Experimental Division of the Caribbean National Forest. El Verde Field Station is on the northwestern flanks of El Yunque, one of the principal peaks of the Luquillo Mountains. The Experimental Forest surrounding the station at El Verde is strictly for research on the properties and processes of the rain forest. *Eleutherodactylus coqui* is the most abundant of eight *Eleutherodactylus* species that inhabit the forest at El Verde. The station is open year-round to research biologists as space and scheduling permit. Information on facilities and research opportunities at the station may be obtained by writing to the Director of Terrestrial Ecology at the above address.

In their native habitat, "coquíes" can be collected most easily during their nocturnal activity period. Males call from fairly exposed elevated perches and are quite accessible. Females, which are silent, are more difficult to locate. Females often climb out of reach on wetter nights, so few may be found near the ground (Stewart, 1985). Daytime collecting, consisting of searching the leaf litter, is less productive in terms of adult animals, but may reward the searcher with a higher incidence of developing clutches of *E. coqui*, usually deposited in the same sort of curled leaves and rolled petioles of the sierra palm (*Prestoea montana*) that frogs use for daytime retreat sites (Townsend, 1984). Males usually brood their eggs from the time of oviposition until hatching, or even several days beyond (Townsend *et al.*, 1984).

Although *E. coqui* is found at high densities in forested habitat, it will readily use marginal habitats, including human-altered ones. Frogs may be collected by searching areas around houses, gardens, or other human-altered habitats that have sufficient vertical substrates or vegetation on which frogs can perch. Densities in such marginal habitats may be lower, and females may be more difficult to locate. An alternative method is to "seed" an area with small enclosed cavities, such as short pieces of PVC pipe or short sections of bamboo stems, which mimic the natural retreat sites of coquíes. Frogs will readily use these for retreats and nest sites if available. Because it requires some amount of time for frogs to discover these new sites and then occupy them, such a technique works best for investigators doing long-term field work or making repeated visits to the field.

Terraria for laboratory maintenance. Eleutherodactylus coqui is an ideal laboratory animal. Individuals of the species can be easily maintained in captivity for several years and will breed in laboratory colonies for up to 1.5 years after collection (Townsend, unpub. data).

Coquíes will live under a variety of conditions, but in our experience, healthy colonies are best maintained in glass aquaria with lids that allow some ventilation. Frogs can be kept in 12–18 gallon aquaria with screen tops that have been partially blocked with plastic wrap or some other transparent waterproof material, the position of which can be adjusted to mediate relative humidity within the tank. Although accustomed to an environment with high relative humidity, coquíes do not tolerate well constant humidity near saturation with little or no air exchange. Periodic wetting and partial drying of laboratory aquaria seem to provide the best conditions for maintaining frogs with maximum vigor and reproductive capacity.

Frogs should not be kept under crowded conditions. A rule of thumb is a minimum of 1.5 gallons of tank capacity per frog. If the object of the research is to encourage breeding, the investigator will want to maximize the number of males and females that can interact while avoiding possible inhibition of calling by males that are too closely spaced. Males maintain a certain minimum nearest neighbor distance in the field, below which they will challenge one another acoustically, or one will stop calling. In such situations, it would be better to keep frogs in larger aquaria or tanks.

Contents of holding tanks may vary, but must include at least two items: one site per frog to be used as a protected daytime retreat site, and a source of water for rehydration. Pieces of 1–1.5 inch diameter PVC pipe or plexiglass tubing, cut into 3–8 inch lengths, serve as excellent retreat sites. Frogs readily adopt these as retreat sites, and males will nest in them as well. If placed in a more or less vertical position in the aquarium, these also serve as elevated perch sites. This species spends a good deal of its active nocturnal period perched above the ground. It is unnecessary to provide standing water for coquíes. Several petri dishes or finger bowls filled with sand, gravel, or a soil mixture that will hold water should be placed in each aquarium. Frogs will sit in these at night and take up water through their ventral abdomen. The bottom of the aquarium may be covered with gravel, sand, or soil to serve as an absorbent medium for urine and a source of water vapor to maintain appropriate humidity within the aquarium. If frogs are to be maintained at fairly low densities, the aquarium may be lined with moss or planted, but such an arrangement makes periodic cleaning difficult. If maintained at higher densities, it is best to cover the bottom with a medium that is more easily cleaned, such as sand or gravel and to add potted plants. Frogs will climb the pots and perch in the potting soil or leaves of the plants. It is best *not* to have standing water in the aquarium bottom. When holding tanks are too wet, either from excessive standing water or stagnant, saturated air, frogs can become edematous, especially in the thighs. This leads to loss of vigor and in some cases, death.

Feeding. Feeding of adult frogs is straightforward. All adults readily eat domestic crickets (*Acheta domesticus*) if the crickets are alive and active. At temperatures between 20–25°C, an individual frog need be fed only 3–4 adult crickets per week. We recommend that crickets be dusted with a mineral or vitamin supplement twice a month to cover possible dietary deficiencies from such a highly restricted diet. Frogs do not feed well on mealworms, and *Drosophila* are generally too small and active to serve as a good food source. Unlike *Gastrotheca*, coquíes cannot be trained to take non-moving food.

Hatchling froglets average 6 mm in size and are extremely difficult to maintain, principally because of difficulty with feeding. *Drosophila* are actually too large, and even wingless forms will hop about too rapidly for the young to catch. A large portion of the diet of these tiny froglets in the wild consists of tiny arthropods including ants, arachnids, beetles, and probably collembolans, all less than 3 mm in length (Townsend, 1985). We have been marginally successful with feeding very small *Collembola* (springtails), but colonies of these insects can be difficult to maintain at sufficient densities to provide a long-term constant food supply.

Reproductive behavior. Because developmental studies may be concerned with very early development, it is important to know the schedule of events around fertilization in *E. coqui.* This species practices internal fertilization (Townsend *et al.*, 1981). Consequently, eggs are already fertilized and on their way to first cleavage by the time of egg laying (Elinson, 1987b). Fertilization seems to occur 1–3 h prior to egg laying. By observing the mating and courtship behavior of *E. coqui,* the investigator can identify with some precision when fertilization is taking place and can be alert for the appearance of eggs or be able to sacrifice females to obtain eggs before laying.

Nocturnally active, male coquíes give advertisement calls to attract gravid females. Calling starts shortly after dusk and often continues into the early morning. In the field, a gravid female approaches a calling male, usually before midnight, and makes contact with him. The male then leads the female to a prospective nest site. If the female enters and remains in the nest site, the pair remain in contact, with the female beneath the male although she is never clasped by him. After 5.5–7.5 h, they adopt a unique position in which the female, while still beneath the male, places her hind legs on top of his (Townsend and Stewart, 1986a). The adoption of this unique "reverse hind leg clasp" (Fig. 3) is a valuable cue to the investigator that the female will soon ovulate, usually within an hour. Males probably inseminate females soon after adoption of the reverse hind leg clasp as well. Ovulation seems to involve major body spasms, culminating in abdominal contractions by the female. These can continue for over two hours before the first eggs are actually laid. It is during the prelaying period of spasms and contractions that freshly fertilized eggs may be obtained by anesthetizing the female and dissecting the eggs from her ovisac, a common junction of the paired oviducts. Because of the long interim period between the female's entry into the nest site and adoption of the reverse hind leg clasp by the pair, it is possible to anticipate laying with some accuracy. Egg laying usually occurs within 8.5–12 h of the pair initially entering the nest, and within 2–3 h of adoption of the reverse hind leg clasp. Because initial contact between a calling male and a gravid female typically occurs during midevening, egg laying usually occurs conveniently between 7 am and noon the following morning. The investigator just has to check the male retreat sites each morning to see if there will be freshly fertilized eggs that day.

Under ideal laboratory conditions, a female coqui produces a clutch of about 40 eggs every 5–8 weeks. Females only deposit clutches following courtship with calling males; attempts to trigger ovulation by injection of hCG or other gonadotropins have not been successful. It appears that ovulation requires the appropriate sensory signals derived from some aspect of courtship, amplexus, or internal fertilization.

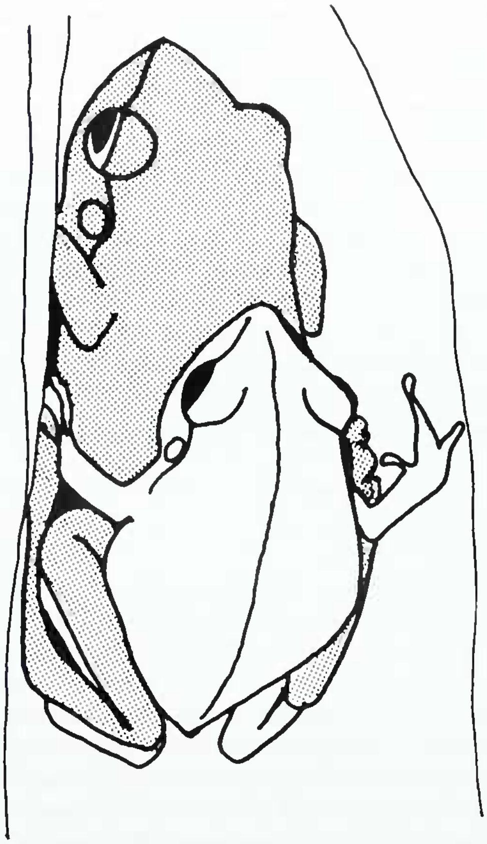

Figure 3. Reverse hind leg clasp during amplexus in *Eleutherodactylus coqui.* This unusual position, with the female's legs on top of the male's legs, is a sign that egg-laying will soon begin. (Drawn from photograph in Townsend and Stewart, 1986a.)

Culture of embryos

The male broods the eggs for about three weeks during which time the eggs develop to hatched froglets (Townsend *et al.*, 1984). The clutch can be removed from the male and his nest site, however, and raised in several ways. A typical culturing method is to place the eggs on absorbent filter paper soaked with spring water in a closed Petri dish. Eggs undergo normal development as long as the filter paper is kept wet, and temperatures are maintained at 21–25°C. Embryos are staged according to Townsend and Stewart (1985).

While development is excellent under these conditions, the embryos are enclosed in their jelly capsules and are not accessible to experimental manipulation. For experimental purposes, embryos can be cultured in water (Lynn and Peadon, 1955) or in 20% Steinberg's solution (Elinson, 1987b). Jellied embryos are submerged in 20% Steinberg's, and the outer and middle jelly layers are removed with watchmaker's forceps. Embryos with intact jelly show retarded development, possibly because of reduced gas exchange. It is initially difficult to remove the fertilization envelope and inner jelly layer, but it becomes easy to do so by Townsend/Stewart stage 7. The embryos continue to develop in 20% Steinberg's until Townsend/Stewart stages 14–15 when they are allowed to crawl onto land. Development of embryos from most clutches occurs normally in 20% Steinberg's, although embryos in some clutches develop edema around Townsend/Stewart stage 7.

Normal development

Eleutherodactylus coqui lends itself to the study of development because of several features. Eggs are large, averaging 4 mm, and are virtually unpigmented for the first two-thirds of development. Many internal features, including the cardiovascular system, nervous system, the development of eyes and ears, and the formation of endolymphatic calcium deposits, are clearly visible in the rather translucent embryo (Townsend and Stewart, 1985). Developmental studies have been conducted on several *Eleutherodactylus* species, including studies by Sampson (1904), Lynn (1942), Gitlin (1944), Lynn and Lutz (1946, 1947), Goin (1947), Jameson (1950), Hughes (1959, 1962), Adamson *et al.* (1960), Chibon (1960), Valett and Jameson (1961), Wake (1978), and Townsend and Stewart (1985). Detailed histological descriptions of organ formation are provided by Lynn (1942), Adamson *et al.* (1960), and Chibon (1962).

Unlike the many normal tables available for *Rana* species, *X. laevis,* and other species with typical embryonic development followed by a free-living larval stage, there are few detailed tables for direct developing anurans. As a first attempt at a general staging scheme to characterize gross external changes in morphology, Townsend and Stewart (1985) proposed a 15-stage scheme that covers development from fertilization to hatching in approximately equal time intervals. This normal table was designed with field studies in mind, so it provides only three stages for the period from fertilization to neurulation. A generalized table for aquatic species (Gosner, 1960) can be used for the preneurula stages. A synopsis of the most distinctive aspects of each of the Townsend/Stewart stages will be mentioned here (Fig. 4).

Stages 1 and 2 cover blastulation and gastrulation, respectively. As with *G. riobambae* and other large-egged amphibians, the blastocoel roof of the *E. coqui* embryo is translucent. Unlike *G. riobambae,* a large, prominent dorsal lip of the blastopore indicates the start of gastrulation in *E. coqui.* The presence of a neural plate and neural folds indicates the onset of stage 3. These first three stages take 3–4 days in *E. coqui* compared to about 2–3 weeks for the comparable development in *G. riobambae.*

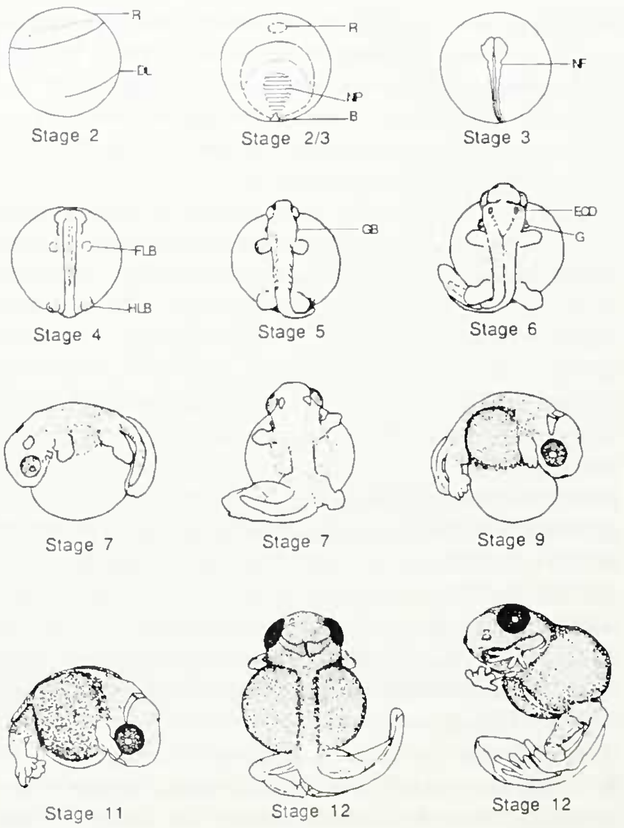

Figure 4. Selected Townsend/Stewart stages of *Eleutherodactylus coqui* development. Gastrulation (Stage 2, lateral view; Gosner Stage 10) is marked by a prominent dorsal lip (DL) of the blastopore, similar to frogs with aquatic development. The embryo, however, is all white with a translucent blastocoel roof (R). A dorsal view of a late gastrula/early neurula (Stage 2/3; Gosner Stage 13) shows the neural plate (NP), an almost closed blastopore (B), and a clear window of uncovered blastocoel roof. Different shades of white allow one to distinguish the neural plate, the rest of the archenteric roof, and the advancing edge of the archenteron. The neurula (Stage 3; Gosner Stage 15) has clear neural folds (NF). Front and hind limb buds (FLB, HLB) form rapidly (Stage 4), and gill buds (GB) become visible (Stage 5). White endolymphatic calcium deposits (ECD) mark Stage 6. The pigmented body wall advances over the yolky cells, the ECD enlarge, and the digits become distinct (Stages 7, 9, 11, 12). The embryo, in its jelly capsule, has a vascularized paddle-shaped tail wrapped around its body to provide a respiratory surface. (Modified from Townsend and Stewart, 1985.)

Stage 4 is distinguished by the paired appearance of pimple-like swellings on the surface of the yolk lateral to the neural tube. These are incipient limb buds. Stage 5 is indicated by early limb buds clearly attached to the main trunk and by the initiation of gill buds. Stage 6 is marked by the first appearance of pigment in the eyes and the first evidence of endolymphatic calcium deposits (ECD) as paired points of pure white material lateral to the junction of midbrain and hindbrain. In stage 7, elbow and knee joints are first evident, and the single pair of gills exhibit their maximum development. Gills are never covered by an operculum in this frog. In stage 8, embryos exhibit the first signs of digits, particularly on hind limbs, and ECD are quadrangular patches with slight anterolateral extensions. From stage 6 to stage 9, the head and trunk of the developing embryo slowly gain pigment. By stage 9, a moderately pigmented body wall surrounds approximately ⅓ of the large yolk supply. Also by stage 9, the single pair of gills has disappeared from external view. Stage 10 is marked by eyes with completely darkened irises while the pupils are still clear. The ECD extend anterolaterally to the rear of the eye but are still separated by a gap medially, and a pigmented body wall surrounds ½–⅔ of the yolk. In stage 11 embryos, toes reach about half of their final length and the pigmented body wall finishes enclosing the yolk reserve. Stage 12 is a longer period, during which the ECD articulate at the embryo's midline and form a broad, shallow U with somewhat broadened base and spread arms. The pigmented body wall completely encloses the yolk, and the pigmentation wall is heavy enough to begin obscuring the ECD at this stage. Stage 13 is marked by full length toes with slight swellings at their tips indicative of incipient toe discs. The first evidence of pigmented eyelids appears, and the initial regression of the unpigmented tail begins. Stage 14 embryos possess toes with full toe discs, eyes with adult coloration (iris golden-brown above, bronze-brown below; pupil black), and clear banding patterns on the hind legs.

Coquí eggs may hatch anytime during stage 15. Embryos at this stage possess full coloration, including any of several distinct pattern morphs exhibited by the species. The remains of the ECD are completely masked by dorsal pigmentation. A bifurcate, black egg tooth at the symphysis of the upper jaw, first evident in stage 12 or 13, is apparently used by the froglet to rupture the egg membrane and hatch.

Development at 25°C requires approximately 17 days (Townsend and Stewart, 1986b). Hatchlings average 6 mm in length, and possess a tail remnant that requires up to two days to resorb after hatching. The large yolk reserve usually lasts for up to a week.

Manipulation of embryos

The normally terrestrial embryos of *Eleutherodactylus* can be cultured in water or in simple salt solutions as described earlier. This permits various experimental manipulations such as surgery or chemical treatments which would otherwise be very difficult.

For instance, Lynn and Peadon (1955) were interested in the role of thyroxine in the development of *E. martinicensis*, a direct developer like *E. coqui*. Thyroxine causes metamorphosis from tadpoles to adults in frogs with aquatic development. Embryos were freed from the jelly and fertilization membrane at Townsend/Stewart stages 6–7, and cultured in tap water with either thyroxine or phenylthiourea, a thyroid inhibitor. Thyroxine caused regression of the tail and degeneration of the pronephros. Limb development, however, was not under thyroid control as it is in metamorphosing amphibians. It is clear that thyroxine and other chemicals given exogeneously can enter these embryos.

Surgery is also possible. Hughes (1962) transplanted limb buds between embryos of *E. martinicensis* to see the interaction between the developing limb and the nervous system. Embryos as early as Townsend/Stewart stage 6 were removed from their jelly capsules and initially cultured in the capsular fluid. A limb bud was cut from one embryo and grafted via a small incision to a second embryo. After the operation, the embryo was allowed to heal for a day in Holtfreter's solution (approximately equivalent to 100% Steinberg's). Thereafter the embryo was cultured in water.

From these limited experiences, it appears easy to experiment on embryos older than Townsend/Stewart stage 6. Whether earlier embryos can be manipulated depends very much on whether they can be removed from the jelly capsule without injury. A second difficulty is that the early embryo may require the structural support provided by the jelly capsule. The use of dishes with an agar base or filled with Percoll (Pharmacia), a high density, low tonicity solution, may permit culturing of early embryos without jelly.

Conclusions

Terrestrial-breeding frogs provide opportunities for analysis by both developmental and evolutionary biologists (Elinson, 1987a, 1990). Obvious comparative and evolutionary questions include:

a. How does large egg size affect early development?

b. How are reproductive adaptations, such as the female's pouch or the male's brooding behaviour, controlled hormonally?

c. What is the cellular and hormonal basis of direct development, an ontogeny without metamorphosis?

d. How did the different reproductive and developmental patterns evolve?

Beyond these questions, embryos from terrestrial-breeding frogs would be ideal for examining certain developmental problems. For instance, the translucent blastocoel roof in both *G. riobambae* and *E. coqui* would allow the migration of internal cells during gastrulation to be followed in intact embryos. The early development of large limb buds in *E. coqui* would permit the analysis of pattern formation in limbs. Finally, the eggs and embryos themselves present unique questions for cell and developmental biologists. Certainly the formation of oocytes with hundreds of nuclei and the controlled degeneration of them is an intriguing problem for the future.

In this article, we have described how to obtain eggs and embryos for the laboratory investigation of the unusual ontogenies found in these frogs. While these recipes permit the investigator to work in Europe or North America, we would urge that anyone interested in these and other such anurans, travel to their country of origin. This would not only allow the observation of the animals in their native habitats, but would also promote the interaction between scientists from temperate and tropical zones.

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The Nocturnal Emergence Activity Rhythm in the Cumacean *Dimorphostylis asiatica* (Crustacea)

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Abstract. The crustacean *Dimorphostylis asiatica* inhabits the sublittoral zone and actively swims in the water at night. Males are positively phototaxic, and can be collected on the surface by using a light at night. The timing of this emergence was investigated in the field. Nearshore collections of males have demonstrated a clear rhythmic pattern, with emergence synchronized with both day-night and tidal cycles. The remarkable feature of this record was that the tidal aspect of the pattern was modified seasonally. While emergence was strongly synchronized with high tide during the winter and spring, tidal synchrony was scarcely detected in summer and autumn. Environmental factors that affect the seasonal modification of the activity pattern are still unknown.

Introduction

On intertidal and estuarine shores, organisms are exposed to periodical changes due to tides and the day-night cycle. These organisms have often evolved, as an adaptation to these environments, timing of activities synchronized with both daily and tidal cycles. The activity patterns differ, reflecting the degree of synchronization with these environmental factors, *i.e.*, daily rhythms (Enright and Hamner, 1967; Hammond and Naylor, 1977), tidal rhythms (Morgan, 1965; Klapow, 1972), and timing with both components (Barnwell, 1966; Benson and Lewis, 1976).

Although seasonal modification of rhythmic behavior is well documented for terrestrial species (see reviews by Saunders, 1976, and Gwinner, 1981), few investigations have been carried out on such aspects of intertidal and estuarine organisms. The present study deals with long-term field observations on the nocturnal crustacean *Dimorphostylis asiatica* inhabiting the nearshore sublittoral zone in Japan. The nocturnal vertical migration of other cumaceans in the field was previously reported by Corey (1970).

Materials and Methods

Adult males of *Dimorphostylis asiatica* (Order: Cumacea), 2–4 mm in body length, living in the Inland Sea of Japan, were studied. The animals hide in the mud flat in the daytime. They swim actively after sunset, and only the males are attracted to light. In spite of many plankton tows, no individuals were caught during the day, so the study focused on nighttime activity. A tungsten torchlamp (100 V, 200 W) was placed on the edge of a floating pier (about 10 m from shore), and the light beam was directed to the surface of the sea. The maximum depth around the collection site is about 3–4 m at the spring high tide. *D. asiatica* individuals swimming under the light can be easily distinguished by their bright yellow color and unique pattern of swimming. As soon as they appeared in the illuminated collection area (about 1 m × 2 m in surface area), the animals were caught with a flat hand net (10 cm in diameter, with mesh size of about 1 mm) and brought to the laboratory.

Collections were made for 30 min every hour from sunset until sunrise, after which few *D. asiatica* individuals were seen near the surface of the water. Following each 30-min collection, the electric torch was turned off. Nightly collections spanning more than two weeks were carried out seven times (4–22 Mar. 1987; 1–16 May 1987; 28 June 28 to 6 Aug. 1987; 11 Sep. to 7 Oct. 1987; 5–20 Nov. 1987; 5–20 Jan. 1988; 3–18 Mar. 1988).

Times of sunset (SS), sunrise (SR), high water (HW1, HW2), and low water (LW1, LW2) were based on data published by the Japan Meteorological Agency.

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* Deceased on 29 October 1988.

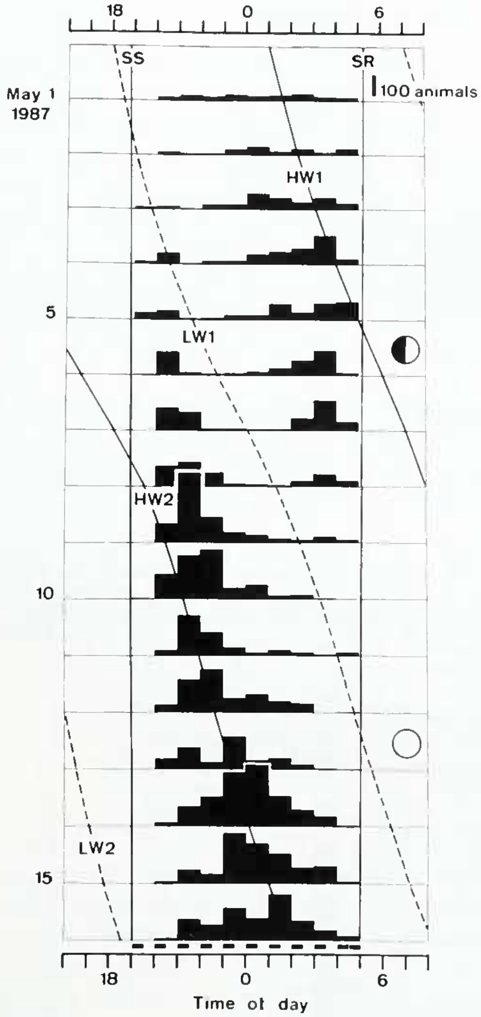

Figure 1. The emergence pattern in May (1–16 May 1987) as determined by the number of *Dimorphostylis asiatica* individuals captured every night from 19:00 to 05:00. The actual times of the collections are indicated by the bars on the time scale. SS: sunset; SR: sunrise; HW1 and HW2: high tides; LW1 and LW2: low tides.

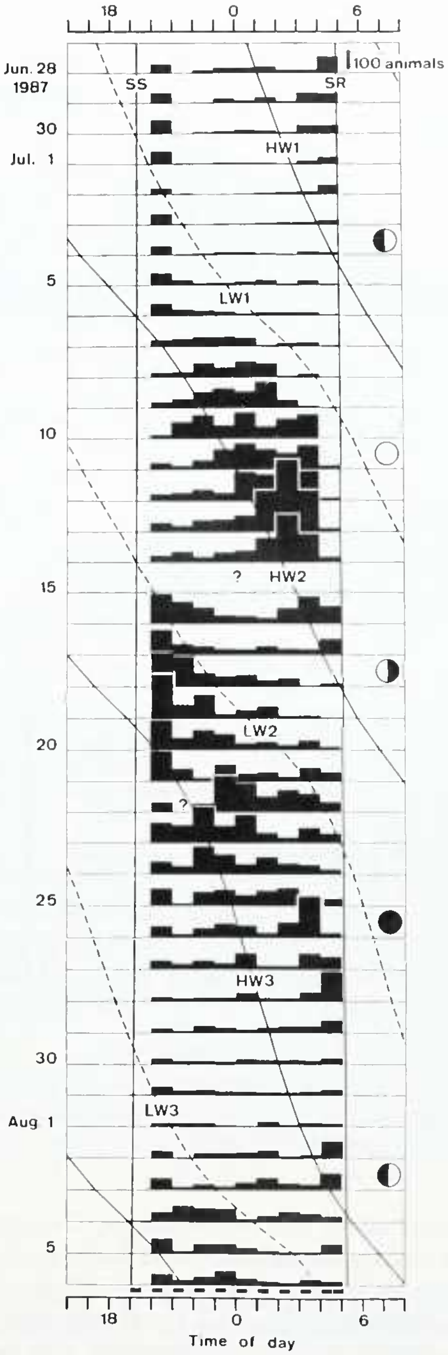

Figure 2. The pattern of *Dimorphostylis asiatica* emergence in July (28 June–6 Aug. 1987). Collections were made from 19:00 to 05:00. ?: indicates that no collections were made on 15 August due to bad weather. Other symbols as in Figure 1.

Results and Discussion

The emergence patterns of *D. asiatica* were summarized in each season.

Emergence pattern in March and May

Figure 1 shows the number of animals collected during each hour of the night in May. Most animals were collected near the time of the night high tide. When high tides (HW1 and HW2) occurred around the time of sunrise and sunset (6–7 days surrounding the first quarter of the moon), the activity exhibited two peaks in the night, one shortly after sunset and another before sunrise. Collections in March showed a similar pattern (data not shown).

Emergence pattern in July

The tide-correlated timing, which was clearly evident in Figure 1, became vague during the summer (Fig. 2). Until 4 July, bimodal peaks of emergence activity ap-

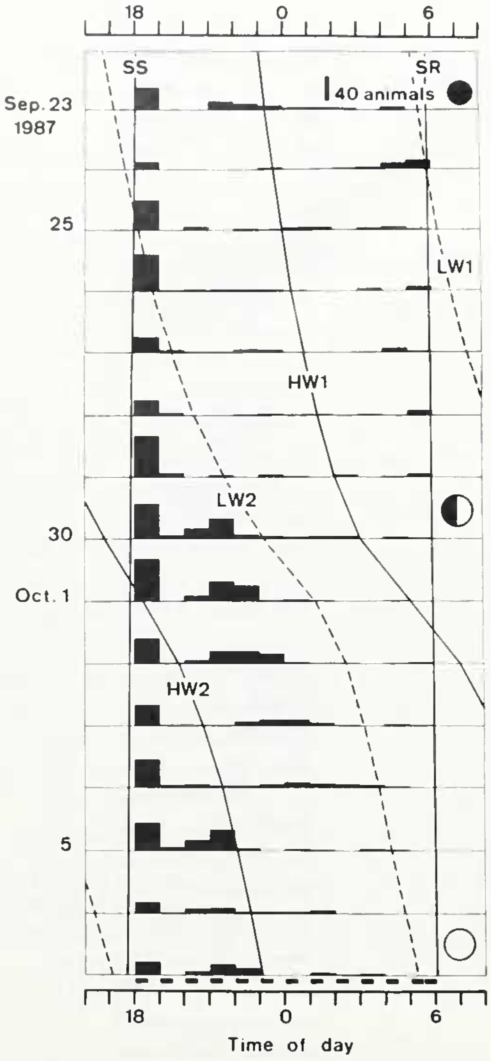

Figure 3. The pattern of *Dimorphostylis asiatica* emergence in September–October (23 Sep.–7 Oct. 1987). Collections were carried out from 18:00 to 06:00. Symbols as in Figure 1.

peared after sunset and before sunrise. Thereafter, those peaks of activity disappeared, and most of the emergence took place on the ebb tide (5–10 July). Compared with the data illustrated in Figure 1, the activity pattern in this season was not as clearly synchronized with the time of high water. For six days surrounding the last quarter moon (18 July), large peaks of emergence were recorded just after sunset. Tidally correlated timing of emergence was weak and disappeared thereafter.

Emergence pattern in September and November

Tidally synchronized timing was scarcely detectable in either the data from September or from November; the pattern is characterized instead by a peak coinciding with the time of dusk (Fig. 3).

The pattern in January

The data obtained in January (Fig. 4) show the reappearance of tidally correlated timing in the emergence activities. Although the tidal component is not strongly distinguished in the first half of the record, it constitutes the main activity in the latter half of the data. Figure 4 further shows that the timing was gradually modified from being dusk-correlated to tide-synchronized. The time difference between sunset and the following activity peak (see the data of 5–14 January) was an hour or two greater in January than at other seasons (Figs. 1, 3).

Number of animals emerging per day through a year

The number of animals that were captured on each night is summarized in Figure 5. There was no clear ev-

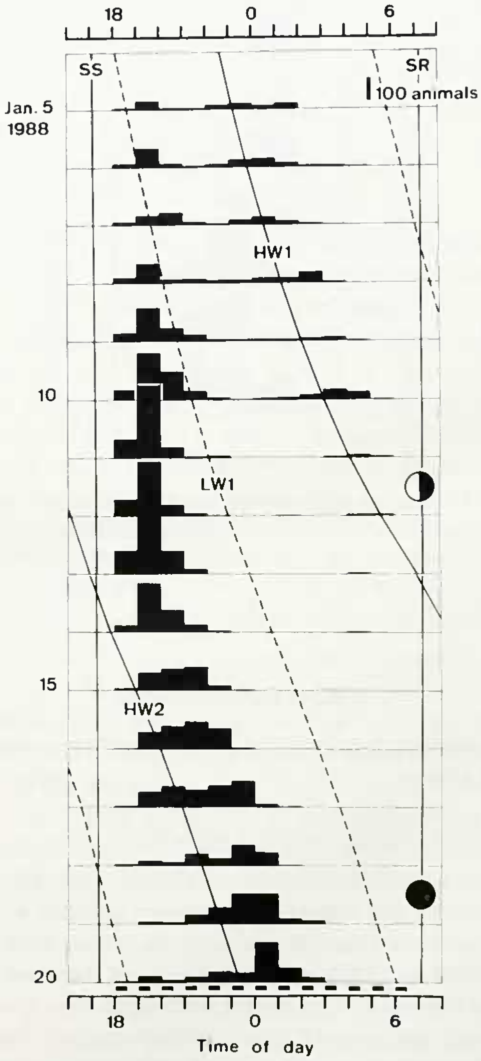

Figure 4. The pattern of *Dimorphostylis asiatica* emergence in January (5–20 Jan. 1988). Collection times were 18:00–06:30. Symbols as in Figure 1.

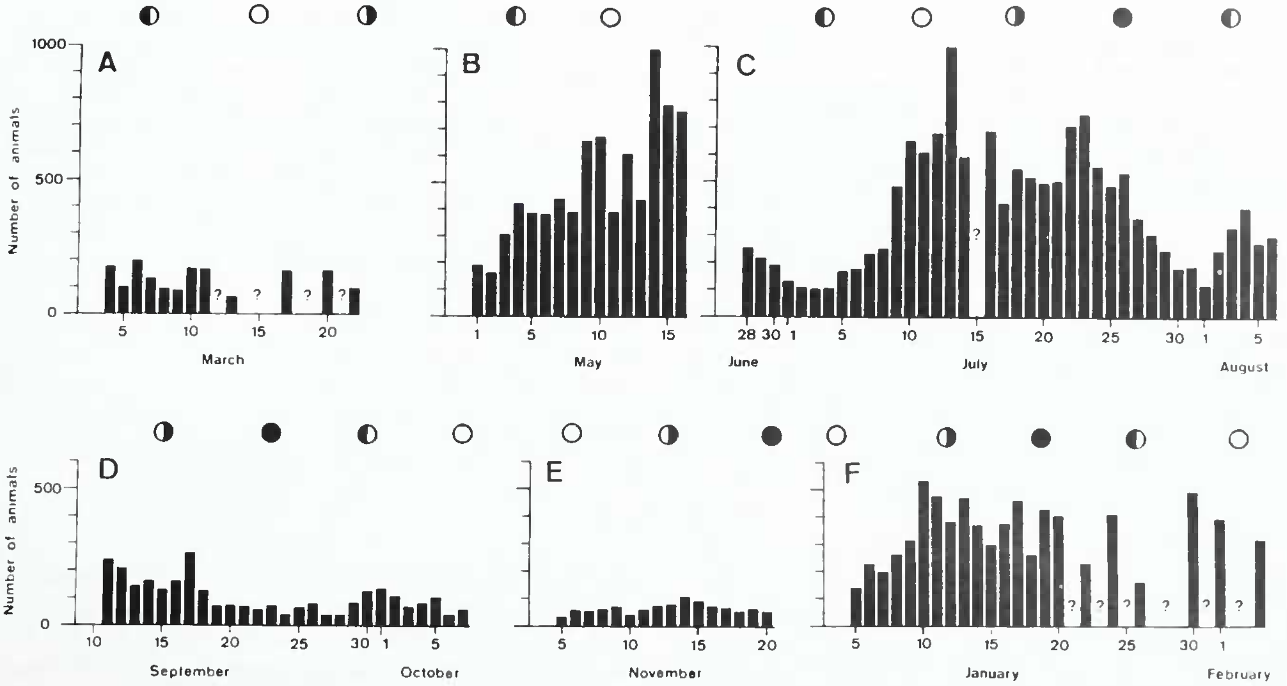

Figure 5. The number of animals captured on each night of collection. A: the data from the first series of investigations; B: the second; C: the third; D: the fourth; E: the fifth; F: the sixth. Abscissa: the dates. Ordinate: number of males. ?: signifies that no collections were made on that date.

idence of lunar or semilunar rhythmic patterns. Although there is some suggestion of a monthly cycle in the record of June–August (Fig. 5C), similar trends are not clearly evident at other seasons.

As indicated by the present observations, a distinct emergence rhythmicity was observed for the male population of *D. asiatica* through the year in the field. The swimming activity was synchronized with environmental day-night and tidal cycles, showing a complex activity pattern. The remarkable feature of the records is the annual modification of the tidal timing involved in the pattern.

To explain such a difference in the activity patterns, it is possible to postulate that the animals synchronize their activity with seasonally changing environmental factors. Intertidal organisms respond to physical factors associated with on-shore tides, *e.g.*, changes of hydrostatic pressure (Enright, 1962; Morgan, 1965; Naylor and Williams, 1984) or cycles of water turbulence (Enright, 1965). If such environmental factors fluctuate seasonally, they might affect the timing of the animals, resulting in the different activity pattern in each season. However, the similar pattern of the fluctuation of tidal amplitude in spring and autumn (not illustrated) makes it difficult to consider that the emergence pattern of *D. asiatica* is directly affected by such factors.

Another possibility is that the internal timing of animals was somewhat different in each season, which might cause the annual modification of the emergence pattern in this population. This possibility is presently under investigation in the laboratory.

Acknowledgments

We thank professor Y. Chiba of Yamaguchi University for his invaluable criticisms of the manuscript.

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Occurrence of Partial Nuclei in Eggs of the Sand Dollar, *Clypeaster japonicus*

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Abstract. Females of *Clypeaster japonicus* bearing eggs with multiple nuclei were occasionally found. DAPI (4′-6-diamidino-2-phenylindole) stained all these nuclei. The summed volume of the two nuclei in binucleate eggs was similar to the nuclear volume in mononucleate eggs from the same batch. On fertilization, two partial nuclei migrated to the center of the egg with a time-course similar to that taken by a single nucleus; they then participated in forming the zygote nucleus, which subsequently formed a single mitotic spindle. These multiple nuclei thus appear to function as genuine nuclei. Possibly they result from the failure of a single nucleus to form during oogenesis.

Introduction

While studying the migration of female nuclei toward the center of echinoderm eggs for syngamy, Hamaguchi and Hiramoto (1986) noticed the occasional occurrence of two female nuclei in single unfertilized eggs of the sand dollar, *Clypeaster japonicus*. On fertilization of such eggs, both nuclei came to the center of the egg and contributed to the formation of a single zygote nucleus, which later underwent the normal cycle of mitosis. Boveri (1918) was the first to observe such "Partialkerne," which occur in a Mediterranean species of the sea urchin, *Parechinus microtuberculatus*. Referring to Boveri's discovery, Chambers and Chambers (1961) called them "partial nuclei."

Working on the early development of *Clypeaster japonicus*, we found, among the sand dollars obtained around the coast near Tateyama Marine Laboratory at Tokyo Bay, a few females bearing eggs with multiple nuclei. Because adults of *Clypeaster* are not abundant at the Laboratory, we relied upon incidental occurrences of such females. This note summarizes our occasional observations of "partial nuclei" during three seasons.

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Observations

Because the unfertilized eggs of *Clypeaster* are transparent, we can easily identify the female nucleus under an ordinary microscope as a large clear spot about 10 μm in diameter. At least 1 in 20 females shed some eggs with more than one clear spot, each apparently representing a nucleus. Hamaguchi and Hiramoto (pers. comm.) have noticed that the rate of occurrence of such females along the coast of Sagami Bay is similar.

Fixing the eggs with methanol-acetic acid (3:1), rinsing three times with phosphate-buffered saline (PBS), and staining them with 4′-6-diamidino-2-phenylindole (DAPI, Sigma, at 0.25 μg/ml in phosphate-buffered saline), we confirmed that these clear spots are genuine nuclei (Fig. 1). The occurrence of multiple nuclei in a batch of eggs from a single female was variable, but typically about 10% are binucleate and 1% are trinucleate. The highest proportion of multiple nuclei recorded thus far was 43%; of these, 37% were binucleate and 6% were trinucleate.

The size of each partial nucleus is not uniform. They are usually smaller than the size of single nuclei, but the summed volume of the two partial nuclei is equal to the volume of a sphere 9 to 10 μm in diameter (Fig. 2), which compares well with the diameter of a single nucleus in unfertilized eggs. The size of eggs bearing partial nuclei is similar to that of mononucleate eggs. Thus the binucleate eggs do not appear to arise by fusion of two eggs, each with a single nucleus. Rather, the partial nuclei seem to derive from the incomplete fusion of karyomeres in the telophase of the second meiosis, as Boveri (1918) had already suggested.

Some minutes after insemination, both partial nuclei start to migrate towards the center of the egg, where they eventually fuse (Fig. 3) to form a single zygote nucleus. Figure 4 illustrates this migration, which is measured as a decrease in the radial distance of the nuclei from the

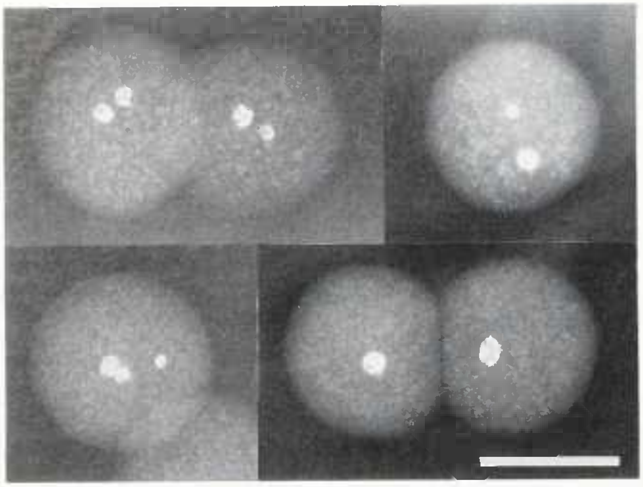

Figure 1. Unfertilized eggs stained with DAPI. Three eggs in the upper row are binucleates. One egg in the lower left is trinucleate. Two eggs (central and right in the lower row) are mononucleates. Scale bar = 100 μm.

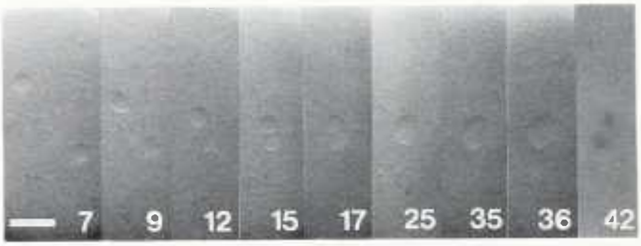

Figure 3. Fusion of partial pronuclei. Numerals are times in min after fertilization. The two pronuclei fused at 17 min. Sperm aster is not clear in this series of pictures. The picture taken at 36 min shows the nuclear envelope starting to break down. At 42 min, polarization-optical observation reveals the anaphase spindle. 26°C. Scale bar = 25 μm.

center of the egg. No difference was detected between the time course of a single nucleus and that of each of partial nuclei. According to Hamaguchi and Hiramoto (1986) two partial pronuclei fuse and then fuse with the sperm nucleus. Fused zygote nuclei formed a spindle (Fig. 3), and the time of the first cleavage was normal. Thus, partial nuclei can only be detected during a short interval of development, and they leave no noticeable trace of their occurrence.

Von Ledebur-Villeger (1972) and Mar (1980) found that the central migration of female nuclei still occurs, even in parthenogenetically activated sea urchin eggs without sperm asters. We confirmed this in *Clypeaster* eggs activated by treatment with 10 μg/ml Ca-ionophore (A 23187) for 2 min (Fig. 5), although the migration started later and was slower (Fig. 6) than that observed for normally fertilized eggs (also noticed by Mar, 1980). In any event, partial nuclei in activated eggs behaved similarly to single nuclei.

Microtubule inhibitors suppress the migration of nuclei towards syngamy (Zimmerman and Zimmerman, 1967; Schatten and Schatten, 1981; Hamaguchi and Hiramoto 1986). Movement of *Clypeaster* nuclei was stopped by treating eggs with 1 m*M* colchicine in seawater 5 min after activation. The movement of partial nuclei was similarly suppressed, and they remained separated, failing to form a single nucleus (Fig. 7). Yet both nuclei eventually exhibited the breakdown and reformation of their nuclear envelopes, as was observed in mononucleate eggs treated with either Colcemid (Sluder, 1979, 1986) or colchicine (Yoneda and Schroeder, 1984).

In several aspects, therefore, the partial nuclei behave very similarly to single nuclei.

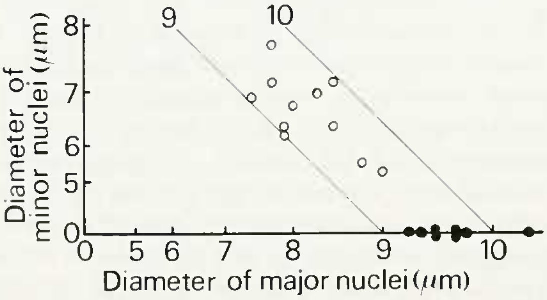

Figure 2. Diameters of partial nuclei measured in living unfertilized binucleate eggs. The diameters of smaller nuclei are plotted (open circles) on a cubic scale against the diameter of larger nuclei also on a cubic scale. Data are from a batch of eggs from a single female. The area between two diagonal lines ("9" and "10") indicates the domain within which the summed volumes of major and minor nuclei is equal to a sphere with a diameter between 9 μm and 10 μm. The diameters of single nuclei from the same batch were the control (filled circles).

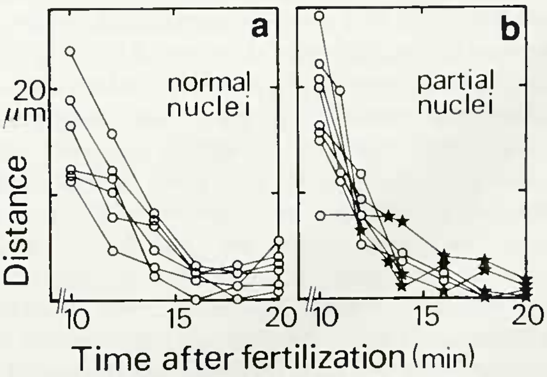

Figure 4. Migration of female pronuclei in normal (a) and binucleate (b) eggs after fertilization at 26°C. Migration indicated by the decrease in the distance (in ordinates) between pronuclei and the center of the eggs. Stars in (b) indicate fused nuclei.

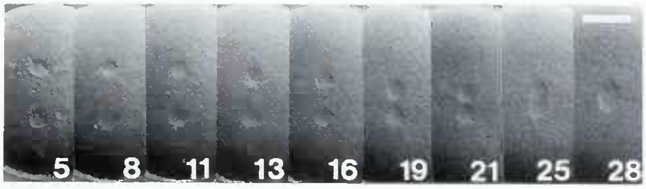

Figure 5. Migration and fusion of two partial nuclei on activation with Ca-ionophore. Times after activation are indicated by numerals. 26°C. Scale bar = 30 μm.

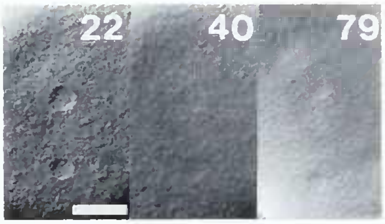

Figure 7. A pair of partial nuclei in activated eggs treated with 1 m*M* colchicine 5 min after activation. Numerals indicate the time after activation. Both nuclei remained separated, but their nuclear envelopes still broke down (40 min) and reformed (79 min). 26°C. Scale bar = 25 μm.

Remarks

Describing the presence of partial nuclei, Boveri's paper (1918) warns us of failure if we enucleate sea-urchin eggs by manual bisection, but overlook the occurrence of multiple nuclei. Thanks to the natural transparency of *Clypeaster* eggs, we can easily detect, with a low power microscope, batches of eggs including those with partial nuclei. Using eggs with partial nuclei may give us some insights into the process of nuclear migration and nuclear fusion in echinoderm eggs.

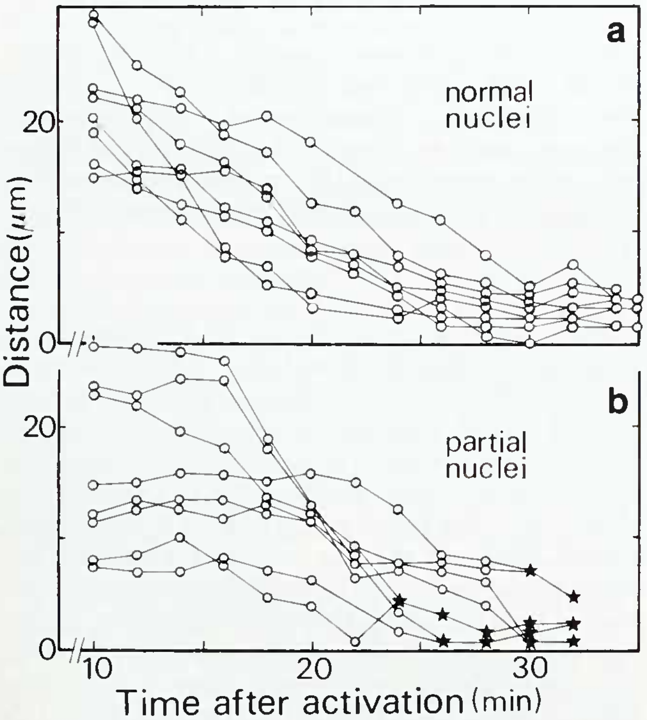

Figure 6. Migration of female pronuclei in normal (a) and binucleate (b) eggs on activation at 26°C, as indicated by decrease in the distance (ordinates) between pronuclei and the centers of the eggs. Stars in (b) mark fused nuclei. Data in Figures 4 and 6 are derived from a single batch. Note that the migration starts later and is slower than that observed in fertilized eggs (*cf.* Fig. 4).

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Drag Coefficients of Swimming Animals: Effects of Using Different Reference Areas

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Abstract. The drag coefficient (C_D) is useful for comparing the hydrodynamic drag among different swimming animals. However, C_D is calculated using an arbitrary reference area for which there is no uniform convention; both total surface area ("wetted area") and maximum cross-sectional area ("frontal area") are widely used. The choice of reference area can have a profound effect on calculations of drag coefficient. To illustrate this problem, drag measurements from two isopod crustacean species were used to calculate C_D based on both wetted and frontal areas. *Idotea wosnesenskii* had a higher mean C_D based on wetted area (0.084) than *Idotea resecata* (0.059), but a lower mean C_D based on frontal area (0.95) compared to *I. resecata* (1.22); both differences are statistically significant. Given that there is no powerful hydrodynamic basis for choosing either reference area, and that conversions between wetted area C_D and frontal area C_D cannot accurately be made for complex shapes, I suggest reporting both wetted area and frontal area C_D's wherever practical.

Introduction

Students of animal swimming often find it useful to measure the hydrodynamic drag experienced by an animal. In steady swimming, thrust equals drag, and data on thrust production are requisite to study such topics as swimming biomechanics (*e.g.*, Webb, 1975; Wu, 1977) and energetic costs of transport (*e.g.*, Hargreaves, 1981; Daniel, 1983). To facilitate comparisons among individuals or among different species, many investigators have borrowed, from engineers, the concept of "drag coefficient" (C_D) defined by:

$$C_D = 2D/\rho v^2 S \qquad (1)$$

where ρ = fluid density, v = speed, S = reference area, and D = drag force (Fox and McDonald, 1978)[1]. The drag coefficient is dimensionless and is typically used to compare the effects of drag on objects of different configurations or morphologies. For a given shape, the drag coefficient is a function of the Reynolds number (Re):

$$Re = \rho v l/\mu \qquad (2)$$

where l = reference length (usually the length parallel to movement or fluid flow), and μ = dynamic viscosity (Hoerner, 1965; Fox and McDonald, 1978). The Reynolds number may be interpreted as a dimensionless index of the relative importance of pressure (inertial or form) drag *versus* viscous (friction) drag (Fox and McDonald, 1978; Vogel, 1981). For simple shapes at $Re \leq 1$, viscous drag predominates, and the drag coefficient is a simple log-linear decreasing function of the Reynolds number. However, at higher Reynolds numbers, pressure drag is most important, and the behavior of C_D with increasing Re is very complex and may be strongly influenced by turbulence (although C_D changes very gradually at $Re \geq 10^6$) (Hoerner, 1965). Most swimming animals have "intermediate" Reynolds numbers (10^2–10^5), where neither viscous nor inertial forces dominate the flow (Hoerner, 1965), and it may be difficult to predict what shapes will give the lowest drag (Vogel, 1981). Biologists have thus found it convenient to compare drag coefficients of a variety of animals to determine which morphologies generate the least drag. For example, Blake (1985) used drag coefficient measurements to show that an actively swimming decapod crab species had a lower drag morphology than two other benthic species. Similarly, Gal and Blake (1987)

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[1] Note that this equation, often given as $D = \frac{1}{2}\rho v^2 S C_D$ [*e.g.*, Hargreaves, 1981; Blake, 1985], defines the "drag coefficient," not the "drag."

compared drag coefficients of a frog species that is entirely aquatic with one that is more amphibious.

Comparisons based on drag coefficients can be complicated by the choice of reference area. The drag coefficient in the form of equation (1) is derived from dimensional analysis (Fox and McDonald, 1978), and the reference area is an arbitrary scale factor with dimensions of $(\text{length})^2$. The choice of reference area can have a significant effect on the magnitude of the drag coefficients. For example, Webb (1975) reported a C_D of 0.015 for a small trout, whereas Nachtigall's beetles had C_D's from about 0.3 to 0.4 (Nachtigall, 1977). Some difference might be expected between fish and beetles on morphological grounds, but the major reason is that different reference areas were used to compute the drag coefficients: the fish drag coefficients were based on total surface area or "wetted" area, C_{D_w} (Webb, 1975), but the drag coefficients of the beetles were based on maximum cross-sectional or "frontal" area, C_{D_f} (Nachtigall, 1977). Both reference areas are commonly used in engineering practice (*e.g.*, Hoerner, 1965; Fox and McDonald, 1978; Bertin and Smith, 1979). Hoerner (1965) and Fox and McDonald (1978) generally use frontal area for the drag coefficients of simple shapes (spheres, cylinders, etc.) and wetted area for streamlined objects and whole vehicles or vehicle models (but without an explicit statement of conventions). Frontal area is typically much easier to measure than wetted area (see below), but wetted area is probably more appropriate in most cases, as animals rarely have simple shapes (if they did, there would be little point in measuring their drag coefficients!). Two other reference areas do have explicit usage conventions: vertically projected area, or "planform" area, is used for the drag coefficient of wings (or other lifting surfaces) and $\text{volume}^{2/3}$ is used for airship drag coefficients (Vogel, 1981).

In the present paper, data from two species of swimming isopods (Alexander, 1988; Alexander and Chen, 1990) are used to show how the choice of reference area can have a profound effect on drag comparisons. Choosing the appropriate reference area in different situations is also discussed.

Materials and Methods

All drag measurements and wetted area measurements are taken from Alexander and Chen (1990). Briefly, specimens of *Idotea resecata* and *Idotea wosnesenskii* (Isopoda: Crustacea) were preserved, fixed in a life-like swimming posture, and mounted on a force transducer; they were placed in a flow tank at a flow speed equal to a realistic swimming speed, and the drag was measured with the force transducer. Wetted area was estimated by approximating animals as oblate spheroids, with body lengths used for major axes, and the means of maximum body height and width used for minor axes (Alexander and Chen, 1990).

The frontal area of these same animals was measured as follows. The preserved isopods were mounted directly head-on in the field of view of a closed-circuit, solid-state Sony video camera equipped with a Nikon 55mm macro lens. The image was displayed on a Burle high-resolution television monitor (38 cm diagonal screen). A 1-cm^2 graph paper grid was also in the field of view of the camera. Each isopod's frontal image was traced onto a plastic transparency sheet, along with the 1-cm^2 grid. The isopod tracing and the area grid were cut out and weighed to the nearest 0.1 mg on an electronic balance, and the weight of the area grid was used to calculate the area of the isopod tracing. Each isopod was traced and cut out three times, and the average weight used to calculate the frontal area; typically, tracings for one individual varied by about 3%, never more than 8%. Drag coefficients were calculated using equation (1). Statistical analyses were based on procedures given in Zar (1984) and the microcomputer version of "Minitab" software.

Results and Discussion

Figure 1a shows the drag coefficients based on wetted area for the two species. *Idotea resecata* had significantly lower C_{D_w}'s than *I. wosnesenskii* ($P < 0.001$, $F_{[1,18]} = 19.38$); the mean C_{D_w} for *I. resecata* was 0.059, *versus* 0.084 for *I. wosnesenskii*. Using the same drag data, but recalculating the drag coefficients using frontal area, Figure 1b shows that the situation is reversed; in this case, *I. wosnesenskii* has a significantly lower mean C_{D_f} (0.95) relative to *I. resecata* (mean $C_{D_f} = 1.22$) ($P < 0.01$, $F_{[1,18]} = 12.6$). The data in Figure 1a and b are from the same animals in the same orientation and posture. The only difference is the choice of reference area.

How can such a seemingly trivial change cause such a drastic reversal in the results? Vogel mentioned that when frontal area is used, "stubbier" (shorter, blunter) shapes should have the lowest drag coefficient, but if wetted area is used, elongate shapes will generally have lower drag coefficients (Vogel, 1981, p. 112). Consider two elongate (not bluff) objects with the same frontal area but differing substantially in length: the short object will typically have lower drag, and thus, lower C_{D_f}. In contrast, if two objects have the same wetted area, but one is shorter, the short one will necessarily be more bulbous or "bluff" and, hence, generally have a larger wake. Where $Re > 1$, the wider wake of the short object is likely to cause more drag and hence, a larger C_{D_w}. As Figure 2 shows, the two *Idotea* species exactly fit these descriptions: *Idotea resecata* is more elongate and has a lower C_{D_w}, whereas *Idotea wosnesenskii* is blunter and shorter, and has a lower C_{D_f}. The startling aspect is that the differences between the species

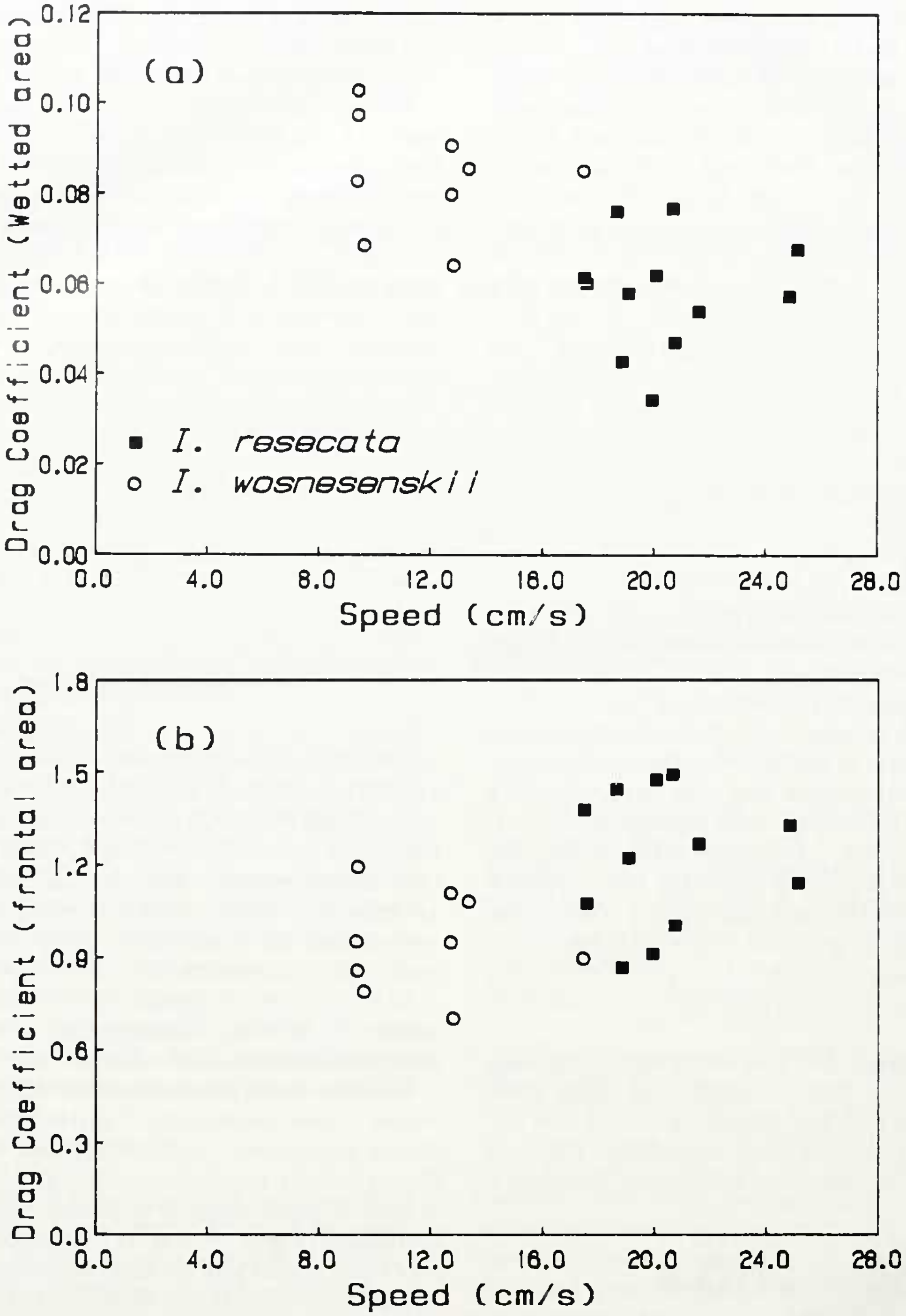

Figure 1. The relationship between swimming speed and drag coefficients for two species of *Idotea*. Each symbol represents a different individual, and shows the mean of 6 to 12 swimming speed trials and 3 drag measurements. The same individuals are represented on both graphs. (a) The drag coefficient calculated using wetted area. (b) The drag coefficient using the same data as in (a) but with drag coefficient calculated using frontal area.

are statistically significant in both cases, even though reversed.

Engineers tend to use the frontal area for drag coefficients where the viscous drag is important, and wetted area where pressure drag is more important (Fox and McDonald, 1978). This is reasonable: at low Reynolds numbers details of shape and orientation have little influence on drag, so frontal area is an adequate scale factor;

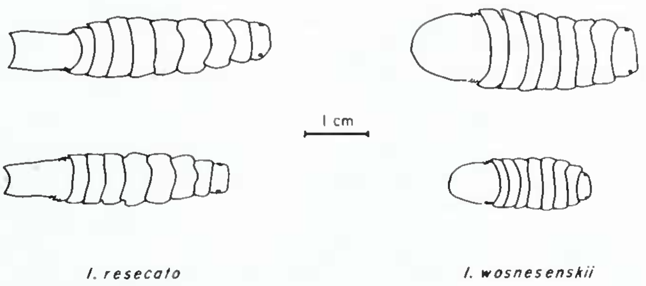

Figure 2. Dorsal views of the bodies of male (upper) and female (lower) individuals of *Idotea resecata* and *I. wosnesenskii* (traced from video images). The mean fineness ratio (length/width) for the individuals in this study was 4.5/1 for *I. resecata* and 2.9/1 for *I. wosnesenskii.*

at high Reynolds numbers, streamlining and orientation are important in that the length of an object in the direction of flow (or movement) affects boundary layer separation and wake size. Thus, at high Reynolds numbers, frontal area would be a poor scale factor, as it would be the same for a sphere and a well-streamlined object. However, the choice of a reference area is ultimately arbitrary, and the typical types of objects to which engineers apply C_{D_f} or C_{D_w} may be as much a matter of convenience, as due to fluid mechanical principles.

The problem for biologists is that many (if not most) macroscopic swimming animals operate at intermediate Reynolds numbers. In such cases, the relationship between drag coefficient and Reynolds number is strongly dependent on the geometry of the object and the presence or amount of turbulence in the fluid (Hoerner, 1965: p. 16.6). Furthermore, one cannot be sure *a priori* whether viscous or pressure drag is most important. Therefore, as Vogel (1981) noted, it is not clear what shapes will give the lowest drag.

Frontal area is attractive simply because it is much easier to measure accurately. Wetted area is difficult to measure on any but the simplest shapes, and virtually impossible on an object as morphologically complex as an arthropod. Thus, for all practical purposes, an estimate for wetted area must be used, as in Cowles *et al.* (1986) or Alexander and Chen (1990). Such wetted area data are very likely to be underestimates for arthropods, as they do not include appendages or surface irregularities due to segmentation; an underestimate of the reference area would lead to an overestimate of the drag coefficient.

Because frontal area will typically be more accurate, its use might seem to be preferable. But, as most animals do not have simple shapes, and as typical swimming animals are elongate in the direction of swimming, wetted area is the reference area of choice. However, the wetted area will necessarily be an estimate (and probably a slight underestimate) for animals with complex shapes, so comparisons among animals with different shapes must be made with due caution. Frontal area is appropriate in some situations, primarily for sessile organisms, or motile organisms with no preferred directionality or which do not move with their longest dimension parallel to the direction of travel.

If a set of drag coefficients is meant as an index for comparing groups of animals, then both C_{D_w} and C_{D_f} should be provided. If a study is meant to investigate the relationship between C_D and some other variable (*e.g.*, Reynolds number or speed) where complete presentation of C_{D_w} and C_{D_f} would be redundant, then future researchers may find the data most useful if major relationships are presented using C_{D_w}, with average or typical C_{D_f} values being included for reference. Drag coefficient data presented without an explicit statement of the choice of reference area are useless; biologists have no clear conventions for choosing reference area.

Other possible reference areas ignored so far in this discussion are planform area and $(\text{volume})^{2/3}$. Planform area is appropriately used when investigating drag on an object that is also generating a significant amount of lift. Such an object will have an additional drag component, induced drag, produced by the same process that generates lift. Indeed, to correctly determine the lift-drag ratio, the planform area must be used to calculate the drag coefficient (Hoerner and Borst, 1975; Blake, 1985). Finally, Vogel (1981) suggests using $\text{vol}^{2/3}$ because, as with lighter-than-air vehicles, the internal volume of a swimming animal is likely to be more biologically relevant than other measures of surface area; also, $\text{vol}^{2/3}$ can be measured as accurately as desired. However, C_D's based on $\text{vol}^{2/3}$ will be lower for blunt shapes than elongate ones (for the same reasons as for frontal area) (Vogel, 1981), which de-emphasizes streamlining. Also, drag coefficients based on $\text{vol}^{2/3}$ are exceedingly rare in the biological literature, so for comparative purposes it may be advantageous to include C_{D_w} data in such studies as well.

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Promotion and Inhibition of Calcium Carbonate Crystallization *In Vitro* by Matrix Protein From Blue Crab Exoskeleton

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Abstract. Soluble organic matrix isolated from dorsal carapaces of the blue crab, *Callinectes sapidus*, inhibited $CaCO_3$ crystallization when free in solution. Immobilized matrix complexes, prepared by crosslinking soluble matrix to decalcified crab carapace, promoted $CaCO_3$ formation in that crystallization in the presence of the immobilized soluble matrix complexes began sooner than in solution controls. In the experimental treatments, deposition of crystals occurred only within the complexes and not in the crystallization solutions. Chitin, a polymer of N-acetyl-D-glucosamine, and chitosan, a deacetylated chitin, which are both insoluble products of the organic matrix of the crab carapace containing little to no matrix protein, did not promote $CaCO_3$ crystallization. Complexes of immobilized polyanionic synthetic peptides on chitosan also promoted $CaCO_3$ crystallization. Addition of a hydrophobic tail (Ala_8) to the polyanionic peptide (Asp_{20}) reduced the rate of promotion, possibly because the hydrophobic tail formed a diffusion barrier around crystal nuclei growth sites, suppressing interactions of nascent crystal nuclei with ions in the bulk solution.

Introduction

The majority of biominerals are formed by an organic matrix-mediated process (Lowenstam, 1981). Organic matrix, extracted by dissolving biomineral in EDTA or dilute acid, is composed of two components that are separated based on solubility. The soluble component—designated soluble matrix (SM)—is typically composed of anionic proteins, and the insoluble component—designated insoluble matrix (IM)—often contains more hydrophobic proteinaceous material that may be crosslinked (Crenshaw, 1972, 1980, 1982; Krampitz *et al.*, 1976, 1983; Weiner, 1979; Weiner *et al.*, 1983; Wheeler and Sikes, 1984; Wheeler *et al.*, 1988a, b). In the crab exoskeleton, the IM may be primarily chitin (Hunt, 1970; Welinder, 1974). The association of soluble and insoluble matrix molecules with each other and with crystals affect how biomineralization is regulated. Of particular interest is inhibition and promotion of crystal growth (Crenshaw, 1982; Mann, 1983; Weiner, 1986; Sikes and Wheeler, 1988a; Wheeler and Sikes, 1984, 1989).

Soluble matrix, when free in solution, inhibits *in vitro* $CaCO_3$ crystallization, but when attached to an insoluble matrix framework it may promote crystallization (Crenshaw *et al.*, 1988; Sikes and Wheeler, 1988a; Wheeler *et al.*, 1988b; Wheeler and Sikes, 1984, 1989; Campbell *et al.*, 1989; Linde *et al.*, 1989). These apparently opposing functions have been explained by the immobilized-matrix/crystal nucleation (IC) hypothesis (Sikes and Wheeler, 1988a; Wheeler and Sikes, 1989), which states that, when matrix is immobilized, it binds to one portion of the surface of the crystal nucleus reducing surficial energy—the energy involved with interactions such as dissolution, or ion exchange, at the surface of the crystal nucleus. This in turn lowers the energy of crystal nucleus formation, the energy required for attaining a stable nucleus upon which crystal growth ensues. If free in solution, however, matrix may inhibit growth of the crystal nucleus, or a crystal surface, by binding to growth sites over the entire nucleus or crystal surface (Sikes and Wheeler, 1988a).

We have designed two *in vitro* systems for evaluation of aspects of the IC hypothesis. One system, referred to as the crab carapace system, enabled us to study the initiation of crystallization by sampling noncalcified and

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calcified cuticle at sequential stages of the molt cycle (Roer and Dillaman, 1984; Roer *et al.*, 1988). In this study, decalcified samples of calcified and noncalcified carapaces at various molt stages from the blue crab, *Callinectes sapidus*, were tested as immobilized matrix complexes in *in vitro* $CaCO_3$ crystallization assays.

The second system used chitosan, a deacetylated chitin. Chitosan provides an insoluble support with reactive amine groups at which various specific matrix molecules can be immobilized. For example, matrix molecules isolated from biominerals have polyanionic and hydrophobic regions. The hydrophobic regions may occur as part of the C-terminus (Schlesinger and Hay, 1977, 1986; Hay *et al.*, 1979; Butler *et al.*, 1983; Rusenko, 1988, Rusenko *et al.*, 1990) or the N-terminus (Gorski and Schimizu, 1988). Anionic regions of matrix molecules may be active sites for crystal binding, whereas the hydrophobic regions may act as diffusion barriers around crystal growth sites (Sikes and Wheeler, 1988a; Wheeler and Sikes, 1989). Therefore, peptide-chitosan complexes were tested as promoters of $CaCO_3$ crystallization, considering that polyanionic peptides having a hydrophobic C-terminus might have a suppressed ability to promote crystal formation.

Materials and Methods

Collection and maintenance of crabs

Blue crabs (*Callinectes sapidus*) were collected from the Mobile Bay area. Some crabs were killed immediately and others were maintained individually in Instant Ocean (specific gravity = 1.005, 22–24°C) and fed chicken liver three to four times per week. Samples of the noncalcified pre-ecdysial cuticle located underneath the old exoskeleton were obtained, as were samples of calcified cuticle after ecdysis. All crabs were staged by the method of Freeman *et al.* (1987).

Crab carapace studies

Dorsal crab carapaces, removed from the crab at various stages, were trimmed of the ragged edges, and cleaned with a soft brush and tap water. Soluble matrix was obtained by grinding approximately five intermolt carapaces (stage $C_{3/4}$) in an electric mill. A slurry of 10 g of powdered material was placed in dialysis tubing (20,000 MW cutoff; Sigma) open to the air to allow the release of carbon dioxide during dissolution of $CaCO_3$. The preparation was dialyzed against 4 l of 2% acetic acid for 24 h, followed by dialysis with two changes of 2 l of deionized distilled water (12 h for each change). Soluble matrix was separated from IM by centrifugation at 30,000 × *g* for 25 min. The SM-containing fraction was adjusted to neutrality. The concentration of protein in SM was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Immobilized matrix complexes were obtained by placing dorsal crab carapaces in 250 ml of 2% acetic acid (pH 2.4) or 10% ethylenediamine-tetraacetate (EDTA) at pH 8.0 for 48 h with magnetic stirring. Decalcification by these methods removed greater than 99% of the calcium, based on atomic absorption analysis (Perkin-Elmer Model 460) of calcified and decalcified samples treated with 1 *N* HCl. The residual calcium was presumably bound to ion exchange sites. Crosslinking of solubilized protein to the samples of carapace was accomplished by including 4% formaldehyde in the decalcification solutions.

Selected decalcified carapaces were boiled in 2 *N* NaOH for 10–15 h until pigmentation was lost. This treatment removed covalently bound protein, leaving only the IM framework, or chitin (Welinder, 1974). The decalcified carapaces were washed in 2 l of deionized distilled water for 48 h with one change of water at 24 h. Carapaces were stored in deionized distilled water. Samples of these carapaces (hereafter referred to as coupons) were cut with a knife blade against a glass plate. The size of the coupons, measured with a ruler as they were cut to be 1.7 cm × 3.0 cm (5.1 cm^2), was chosen to provide more than one coupon per carapace and so that a coupon would fit in the reaction vessel. Coupons were stored in deionized distilled water in Petri dishes.

The solutions that had been used for decalcification and crosslinking SM onto the carapace, as well as controls consisting of unused solutions, were dialyzed against distilled water with 1000 MW cutoff dialysis tubing, and then lyophilized (VirTis freeze dryer). Protein per milligram lyophilized material was determined by the method of Lowry *et al.* (1951). The amount of solubilized protein that was crosslinked to the carapace was determined as the difference in the protein left in the formaldehyde-containing solutions compared to those without formaldehyde.

Peptide synthesis

Peptides used in this study were made with an automated solid-phase peptide synthesizer (Applied Biosystems Model 430A) as described by Sikes and Wheeler (1988b). They were synthesized with the alanine residues located at the C-terminus; thus, the peptides were crosslinked to chitosan (as described below) at the N-terminal aspartic acid residue via glutaraldehyde, so that the C-terminal hydrophobic tail (Ala_8) would be free to interact with the crystallization solution. Sequences of chosen peptides were confirmed by amino acid analysis using a PICO-TAG amino acid analysis system (Waters) with a Varian VISTA 5500 High Performance Liquid Chromatography system. The synthetic peptides were also sequenced with an automated protein sequencer (Applied Biosystems Model 477A), with an on-line amino acid analyzer (Applied Biosystems Model 120A) using standard cycles of Edman degradation, with the amino acid residues identified as phenylthiohydantoin (PTH) derivatives (Applied Biosystems).

Peptide-chitosan studies

Chitosan was prepared by refluxing decalcified crab carapaces at intermolt (stage $C_{3/4}$) in 12.5 *N* NaOH (approximately four trimmed carapaces in 140 ml) for 27 h (Wu and Bough, 1978). The chitosan pieces were then washed in 2 l of deionized distilled water for 48 h, with one change at 24 h, and were stored in deionized distilled water. Coupons of chitosan were also cut with a knife blade against a glass plate and stored in deionized distilled water in Petri dishes. Chitosan was quantified as the percent free amine (wt/wt) of chitosan, as determined by base titration (Filar and Wirick, 1978).

Peptides were crosslinked onto chitosan coupons following the method of Masri *et al.* (1978). Peptides were added to 22 ml of 0.05 *M* sodium acetate at pH 5.80 ± 0.02 in a 30 ml screwcap bottle in a water bath maintained at 20.00 ± 0.02°C (VWR 1155 Constant Temperature Circulator) at a concentration of 100 μg peptide/ml. A chitosan coupon (5.1 cm^2) and 70.4 μl of 2.5% glutaraldehyde (Sigma) were added, and these components were gently stirred for 1 h. This quantity of glutaraldehyde successfully immobilized the peptides without rendering the coupon too brittle for later manipulation. The coupon was tied with a string in a cheesecloth bag and rinsed in two changes of 2 l of deionized distilled water (12 h for each change). Coupons were stored in Petri dishes. The concentration of the peptide in a 2-ml sample of the crosslinking solution, before and after the procedure, was determined by the hydrolysis/ninhydrin assay (Stewart and Young, 1984). The difference between the samples reflects the amount of peptide crosslinked onto chitosan. Control experiments were performed as described above without chitosan coupons.

$CaCO_3$ crystallization assay

Crystal formation was monitored by the change in pH of a solution supersaturated with respect to calcium and carbonate. The assay began with an induction period of relatively constant pH, during which crystal nucleation occurs, and then continues with a rapid decrease in pH indicating crystal growth. The pH was continuously monitored (Orion 901 pH meter) and recorded (Perkin-Elmer, Model 56 chart recorder), with periodic manual notation of exact pH values on the curves. The incubation medium included 29.1 ml of artificial seawater (ASW: 0.5 *M* NaCl and 0.011 *M* KCl) in a round bottom flask placed in a recirculating water bath (VWR 1155 Constant Temperature Circulator) maintained at 20.00 ± 0.02°C, to which 0.3 ml of 1.0 *M* $CaCl_2 \cdot 2H_2O$ was added to obtain 10 m*M* calcium. Next, SM (Lowry protein assay) or peptide was added and stirred for 10 min. Finally, 0.6 ml of 0.4 *M* $NaHCO_3$ was added to yield 8 m*M* dissolved inorganic carbon (DIC), and the solution was titrated to a pH of 8.30 ± 0.02 with microliter quantities of 1 *N* NaOH to begin the assay.

The above system was modified to establish longer induction times to be used for testing promotion of crystallization. A coupon, patted dry three times with a lab tissue (Kimwipe) and weighed (wet weight), was suspended in 53.84 ml of ASW in a round bottom flask at 20.00 ± 0.02°C (VWR 1155 Constant Temperature Circulator). The coupon was suspended by a string attached to a brass pin that held the coupon. Then, 0.55 ml of 1.0 *M* $CaCl_2 \cdot 2H_2O$ and 0.61 ml of 0.5 *M* $NaHCO_3$ were added to a concentration of 10 m*M* calcium and 5.5 m*M* DIC, respectively. The coupon was carefully placed in the solution so that it was not in contact with the electrode or the stir bar. As above, the pH was raised to 8.30 ± 0.02 to begin the experiment.

Verification of $CaCO_3$ deposition

The amount of calcium deposited on coupons, before and after the crystallization experiments, was measured by atomic absorption spectrophotometry (Perkin-Elmer, Model 460). Coupons were removed from the crystallization solution and vortexed in 10 ml of 1 *N* NaOH to remove extraneous calcium, while preventing dissolution of the $CaCO_3$ present on the coupons. The coupons were allowed to air dry before they were vortexed in 10 ml of 1 *N* HCl. The coupons were then dried at room temperature and weighed (dry weight). The acid solutions were then aspirated through an air/acetylene flame, the calcium detected with a calcium lamp, and the concentration determined from a standard curve.

The amount of carbonate removed from solution, which resulted in the observed downward pH drift during crystallization, was calculated using the equilibrium equations for dissolved inorganic carbon (Stumm and Morgan, 1981, pp. 186 and 197). These amounts were compared to the measurements of calcium deposition by atomic absorption spectrophotometry.

In addition, the presence of crystals on the coupons was verified visually by polarized microscopy. Pieces of coupons were viewed under polarized light before and after crystallization experiments, and with and without the addition of 0.01 *N* HCl to cover the samples and dissolve any $CaCO_3$ crystals that may have formed.

Table I

Inhibition of $CaCO_3$ crystallization by crab soluble matrix added at 0.10 μg/ml, and by various peptides added at 0.05 μg/ml

	n	Induction period (min)	Maximum growth rate (pH/min)
Control	14	6.4 ± 1.0	0.050 ± 0.007
Extract 1	5	109.0 ± 57.2	0.046 ± 0.006
Extract 2	9	43.8 ± 11.6	0.053 ± 0.004
Extract 3	3	49.6 ± 10.0	0.058 ± 0.004
Asp_{20}	3	274.6 ± 42.7	0.009 ± 0.012
$Asp_{20}Ala_8$	6	270.0 ± 76.4	0.006 ± 0.006
Asp_{40}	3	298.0 ± 79.2	0.009 ± 0.012
$Asp_{40}Ala_8$	3	290.0 ± 69.3	0.003 ± 0.002

Extracts 1, 2 and 3 were prepared separately from different samples of Stage $C_{3/4}$ carapaces.

Means ± standard deviations.

Statistics

Statistical analysis of data was performed using Truc Epistat (Tracy L. Gustafson, Epistat Services, Richardson, Texas).

Results

Soluble matrix from the blue crab carapace inhibited $CaCO_3$ crystallization, as indicated by a longer induction period (Table I). The inhibitory activity of the extracts varied, possibly reflecting the differing relative amounts of the various components of the crude preparations and the variation in the individual carapaces used for each isolation. In fact, gel chromatography revealed that these preparations differed in average relative molecular weight (Gunthorpe, 1989). Polyaspartate of 20 and 40 amino acid residues, and their counterparts with an hydrophobic (Ala_8) tail located at the C-terminus, inhibited $CaCO_3$ crystallization.

Promotion of crystallization, indicated by a shortened induction period, was shown when coupons of various decalcified carapaces were suspended in the crystallization solution (one-way ANOVA, $P < 0.01$, Table II) with the exception of pre-ecdysial cuticle. Soluble matrix was crosslinked to the carapace during decalcification in the presence of formaldehyde. There was a difference of 5.89 ± 1.08 mg protein/mg calcified carapace (S.D., n = 8; Stage D) and 6.40 ± 0.50 mg protein/mg calcified carapace (S.D., n = 6; Stage $C_{3/4}$) between the decalcification so-

Table II

The effect of fixation on promotion of $CaCO_3$ crystallization by coupons (5.1 cm^2) of decalcified crab carapaces at various stages of the molt cycle

Treatment	n	Wet weight (mg)	Dry weight (mg)	Induction period (min)	Maximum growth rate (pH/min)
Control	28	—	—	16.2 ± 2.9	0.0280 ± 0.0040
Formaldehyde					
0.074%	3	—	—	21.8 ± 5.1	0.0280 ± 0.0030
Chitin	3	221.0 ± 88.6	43.5 ± 14.0	22.3 ± 7.5	0.0280 ± 0.0020
2% acetic acid					
Stage D	4	258.5 ± 43.5	46.8 ± 10.0	9.3 ± 4.2	0.0024 ± 0.0012
Stage $C_{3/4}$	2	280.0 ± 19.0	55.0 ± 2.8	4.8 ± 1.4	0.0029 ± 0.0001
New cuticle					
Pre-ecdysial	3	38.0 ± 8.2	4.6 ± 0.3	15.3 ± 0.3	0.0005 ± 0.0001
Post-ecdysial	5	56.8 ± 3.0	9.5 ± 1.4	2.5 ± 1.0	0.0028 ± 0.0005
4% Formaldehyde in 2% acetic acid					
Stage D	5	225.7 ± 25.9	42.5 ± 3.9	4.5 ± 2.2	0.0035 ± 0.0070
Stage $C_{3/4}$	3	229.1 ± 18.4	64.3 ± 0.5	3.5 ± 1.4	0.0027 ± 0.0030
New cuticle					
Pre-ecdysial (F)	3	57.6 ± 1.5	11.8 ± 0.6	1.6 ± 0.8	0.0046 ± 0.0009
Post-ecdysial	5	55.6 ± 10.2	12.0 ± 3.3	1.6 ± 0.8	0.0032 ± 0.0006
10% EDTA					
Stage D	4	183.8 ± 34.5	28.4 ± 4.5	5.8 ± 1.1	0.0024 ± 0.0001
4% Formaldehyde in 10% EDTA					
Stage D	2	219.9 ± 9.5	42.0 ± 6.9	2.1 ± 0.1	0.0037 ± 0.0010

Postecdysial = Stage A-B cuticle at the leathery phase before becoming brittle with $CaCO_3$.

Stage $C_{3/4}$ = intermolt stage.

Stage D = calcified cuticle covering the pre-ecdysial cuticle.

(F) = 4% formaldehyde only.

Means ± standard deviations.

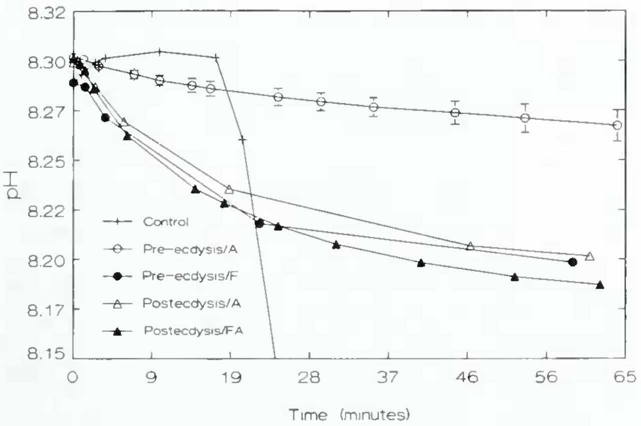

Figure 1. Promotion of $CaCO_3$ crystallization by coupons cut from new cuticle of blue crabs at pre-ecdysis and postecdysis (Stage A–B), following decalcification in either 2% acetic acid (A), 4% formaldehyde (F) to crosslink soluble proteins, or 4% formaldehyde in 2% acetic acid (FA). Crystallization was measured as a downward pH drift, reflecting the removal of CO_3^{2-} from solutions of supersaturated artificial seawater. Promotion of crystallization was indicated by a reduction of the induction period prior to the pH decrease in control solutions. Error bars represent typical standard deviations (n = 3).

lutions with and without formaldehyde. However, there was no significant difference in the ability of coupons to promote crystallization. The ability of coupons to promote crystallization was reduced with prolonged storage (data not shown). Acid-washed pre-ecdysial cuticle did not promote crystallization, presumably because there were few immobilized matrix proteins present. In contrast, crosslinking of soluble protein on pre-ecdysial cuticle promoted crystallization equal to that of formaldehyde-treated or acid-washed postecdysial (stage A–B) cuticle of comparable dry weight (Fig. 1). Coupons of chitin, which should not have protein, did not promote crystallization (Table II), indicating that the protein components may function as nucleating sites.

That the downward pH shifts during the crystallization assays did indeed indicate growth of $CaCO_3$ crystals was verified in three ways: by measurements of calcium on the coupons, by comparison of these measurements to calculations of the amount of carbonate removed from solution, and by the presence of birefringence on the coupons that was acid labile. For example, the coupon that was treated in 4% formaldehyde in 2% acetic acid (Stage D) (Table II) had 20.7 μmole Ca^{2+} deposited on it after crystallization. This compared very well with 20.6 μmole CO_3^{2-} calculated as removed from solution in that same experiment. Finally, birefringence that was present on the coupons after the crystallization experiments was lost following treatment of pieces of coupons with 0.01 N HCl, which is consistent with dissolution of $CaCO_3$ crystals.

When a crystal-promoting coupon was suspended in the crystallization solution, the rate of crystal growth was reduced approximately ten fold relative to controls without coupons. The bulk solution was visibly turbid with crystals in controls, but when a coupon was suspended in the solution, crystals were not visible in the bulk solution. SM from crab carapace, when free in solution, inhibited crystallization (Table I); thus, when present in the coupons, some of it may have diffused into the solution, reducing crystal growth rates. In addition, the lowering of the pH of the bulk solution, due to the growth of crystals on the coupons, would itself lead to a lack of precipitation in the bulk medium. The lack of bulk precipitation was not due to formaldehyde diffusing from coupons in that high doses of formaldehyde, when free in solution, did not inhibit crystallization (Table II).

Coupons of chitin or chitosan, which presumably had no protein, did not suppress crystallization in the bulk solution (Tables II, III, respectively). However, Gunthorpe (1989) demonstrated that small coupons of acid decalcified carapace and the membranous layer that underlies the cuticle inhibited crystallization. But when these coupons were extensively washed in distilled water, some

TABLE III

Effect of coupons (5.1 cm²) of chitosan, glutaraldehyde-treated chitosan, and various peptide-chitosan complexes on $CaCO_3$ crystallization

Complex	n	μmole protein on coupon	Wet weight (mg)	Dry weight (mg)	Induction period (min)	Maximum growth rate (pH/min)
Control	25	—	—	—	15.4 ± 2.5	0.0300 ± 0.0040
Chitosan	5	0	230.4 ± 67.6	71.8 ± 16.6	18.8 ± 4.7	0.0230 ± 0.0060
PolyAsp (MW 11,500)	3	0.040 ± 0.011	182.9 ± 45.4	65.4 ± 10.5	2.9 ± 1.8	0.0036 ± 0.0002
Asp_{20}	4	0.373 ± 0.033	237.4 ± 56.7	64.3 ± 16.7	1.8 ± 0.9	0.0056 ± 0.0009
$Asp_{20}Ala_8$	3	0.320 ± 0.080	189.0 ± 35.9	63.4 ± 14.6	3.1 ± 0.3	0.0042 ± 0.0008
Asp_{40}	4	0.152 ± 0.040	172.0 ± 25.9	57.4 ± 8.2	5.7 ± 1.0	0.0026 ± 0.0001
$Asp_{40}Ala_8$	3	0.126 ± 0.012	180.8 ± 22.0	61.5 ± 2.0	6.2 ± 0.9	0.0022 ± 0.0008

Values are given as mean ± standard deviation for n coupons.

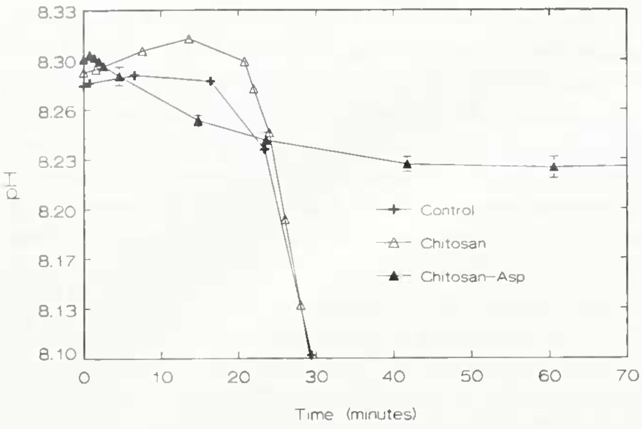

Figure 2. The effect of coupons (5.1 cm^2) of chitosan (without crosslinked peptide or protein) and a peptide-chitosan complex (polyaspartate, MW 11,500, immobilized on chitosan by glutaraldehyde) on $CaCO_3$ crystallization. Error bars represent typical standard deviations (n = 3).

promotion of crystal growth was observed with the washed decalcified carapace present compared to the nearly control levels of crystal growth observed with the washed membranous layer present (Gunthorpe, 1989). Thus, loosely associated protein in the crystal-promoting coupons may have diffused into the solution and inhibited crystal growth. Deposition of crystals was localized on insolubilized protein complexes, giving rise to a slow rate of crystallization that began significantly sooner than observed in controls.

Suspensions of chitosan, a partially cationic surface at pH 8.3, did not promote $CaCO_3$ crystallization (Table III). Various immobilized peptide-chitosan complexes promoted crystallization (Table III; Fig. 2). The presence of an Ala_8 tail on Asp_{20} showed suppression of promotion ($P < 0.01$; balanced, incomplete block design), but this tail did not affect promotion when attached to Asp_{40} (Fig. 3).

The quantities of the various peptides crosslinked to chitosan were statistically the same. Control solutions containing peptides but not chitosan coupons indicated a 4.2 ± 1.6% decrease in the amount of peptides (n = 3) due to interactions with glutaraldehyde after the crosslinking procedure. When the coupon was present, there was a 28.2 to 65.1% decrease in the amount of peptide left in the solution after 1 h. The larger peptides were crosslinked to chitosan to a lesser extent than were the smaller peptides.

As also seen in the studies using the crab carapace system (Table II and Fig. 1), the rate of crystal growth was reduced when coupons with immobilized peptides were suspended in the solution. Crystallization did not occur in the bulk solution in these experiments, rather crystal formation as determined by pH change occurred only on the coupons. This was confirmed by the absence of a pellet of crystals produced by centrifugation of a sample of the bulk solution at 735 × *g*. An equivalent analysis of the bulk solution in control treatments without coupons produced a white pellet of $CaCO_3$ crystals.

Discussion

Inhibition by soluble matrix and by synthetic analogs

Soluble matrix isolated from dorsal crab carapaces, and synthetic peptide analogs of matrix, inhibited $CaCO_3$ crystallization when free in solution. Organic matrices isolated from other organisms are also inhibitors of $CaCO_3$ crystallization when free in solution (Termine *et al.*, 1980; Borman *et al.*, 1982; Doi *et al.*, 1984; Sikes and Wheeler, 1983, 1986; Swift *et al.*, 1986; Wheeler *et al.*, 1988a, b; Wheeler and Sikes, 1984, 1989). Inhibition has been correlated with the affinity of matrix molecules for crystal surfaces and adequate coverage of growth sites on crystal surfaces (Aoba *et al.*, 1984; Wheeler and Sikes, 1989).

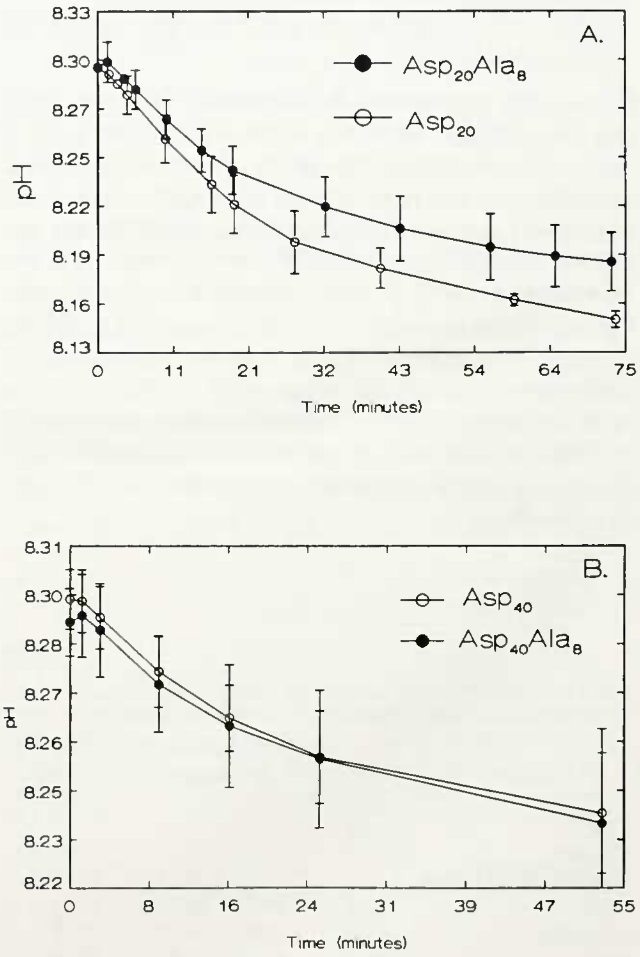

Figure 3. The effect of addition of a polyalanine tail at the C-terminus on polyaspartates of different sizes (A, Asp_{20}; B, Asp_{40}) on promotion of $CaCO_3$ crystallization by immobilized peptide-chitosan complexes.

Immobilized matrix complexes

Decalcified dorsal carapaces of the blue crab (immobilized matrix complexes) and immobilized peptide-chitosan complexes shortened induction periods for crystallization *in vitro*. Shortened induction periods were also observed in collagen systems (Endo and Glimcher, 1988), and similarly, apatite crystals were formed within a shorter period in the presence of proteolipids (Boskey *et al.*, 1988). Shortened induction periods are often considered indicative of an increased rate of nucleation, which can result from a reduction of the energy that is required for nuclei formation (Garside, 1982). A likely cause of such a decrease in nucleation free energy would be a decrease in surficial energy of the nuclei (Wheeler and Sikes, 1989) by the immobilized matrix complex. Crystal nuclei binding by immobilized matrix complexes have been demonstrated (Termine *et al.*, 1981a, b; Addadi and Weiner, 1985, 1986).

The immobilized matrix complex is thought to be formed by an association of soluble matrix with insoluble matrix. The IM framework of crab carapaces is chitin, which, when suspended in a solution supersaturated with respect to calcium and carbonate ions, did not promote crystallization. Further, a derivative of chitin (chitosan) did not promote crystallization when suspended in the solution. With decalcification by acetic acid or EDTA, protein remains associated with the chitin framework (Hunt, 1970; Welinder, 1974; Muzzarelli, 1977; Brine and Austin, 1981). As demonstrated, these latter complexes promoted crystallization. An immobilized matrix complex (whole matrix) from *Nautilus* induced mineral deposition, whereas a preparation of the IM framework alone did not (Greenfield, 1987). To further clarify that SM is the functional molecule, SM was crosslinked to various solid supports. Mineral induction was demonstrated by phosphoproteins crosslinked to collagen (Boskey *et al.*, 1988; Endo and Glimcher, 1988; Linde and Lussi, 1988) or AH-sepharose beads (Lussi *et al.*, 1988; Linde *et al.*, 1989). Soluble matrix from *Mytilus californianus* immobilized on polystyrene films also induced mineral on the film (Addadi *et al.*, 1987). Mineral induction was demonstrated by IM frameworks of other organisms, but the presence of SM was not clarified (Bernhardt *et al.*, 1985; Watabe *et al.*, 1986).

The relative proportions of matrix that was free in solution and that immobilized on the IM framework may correlate with the amount of crystal deposition observed in these studies. Lussi *et al.* (1988) demonstrated that induction by immobilized rat dentin phosphoproteins on AH-sepharose beads can be fully inhibited at high concentrations (>160 μg/ml) of the phosphoprotein when added before the experiment began. A similar phenomenon may, in part, explain the temporal control of calcification in the new cuticle of blue crabs during the molt cycle. The pre-ecdysial layers are not calcified, and only the epicuticle layer is tanned (Roer and Dillaman, 1984; Freeman and Perry, 1985). It may be that the pre-ecdysial cuticle contains more unbound protein that inhibits crystallization, preventing calcification. In contrast, crosslinking of protein onto chitin fibers after ecdysis may promote calcification of the postecdysial cuticle.

Accordingly, pre-ecdysial cuticle that had been acid washed thereby removing SM, did not promote crystallization. With immobilization of pre-ecdysial SM proteins by formaldehyde, crystallization was enhanced. In contrast, postecdysial cuticle promoted crystallization significantly, regardless of the treatment. Roer *et al.* (1988) demonstrated that formaldehyde-treated pre-ecdysial cuticle of fiddler crabs (*Uca pugilator*) induced crystallization, but not to the extent of formaldehyde-treated postecdysial cuticle. Control of calcification was hypothesized to occur by an alteration of the organic matrix resulting in the removal of blocked nucleating sites at ecdysis (Roer *et al.*, 1988).

The immobilized matrix complex can be further characterized by studying specific, synthetic analogs immobilized on a natural IM framework such as chitosan. In so doing, functional groups can be identified. Cationic groups, such as the free amine groups of chitosan, did not promote crystallization. Anionic groups, such as carboxyl groups of aspartate residues, promoted crystallization when immobilized on chitosan supports. In contrast, Addadi *et al.* (1987) demonstrated that polyaspartate ($M_r = 6000$) adsorbed on polystyrene films did not nucleate $CaCO_3$ crystals on the films, perhaps due to differences in the amounts of polyaspartate immobilized and spatial relationships between the polyaspartate and the different solid supports used in the different studies. However, Addadi *et al.* (1987) also showed that blocking carboxyl groups of protein assemblages from mollusc shells reduced the amount of crystals induced on polystyrene films, and presented other evidence for cooperative influences of SO_4^{2-} and carboxyl groups in promoting crystallization. The importance of anionic groups such as sulfate and phosphate have also been discussed in other studies (Greenfield *et al.*, 1984; Endo and Glimcher, 1988).

In addition to the polyanionic regions, matrix proteins frequently contain substantial hydrophobic domains (Schlesinger and Hay, 1977; Hay *et al.*, 1979; Butler *et al.*, 1983; Schlesinger *et al.*, 1986; Gorski and Shimizu, 1988). The hydrophobic domains are required for complete activity of the proteins as inhibitors of crystallization (Hay *et al.*, 1979, Aoba *et al.*, 1984; Aoba and Moreno, 1990). Therefore, Sikes and Wheeler (1988b) evaluated the effect of attaching a polyalanine domain onto polyaspartate molecules of 15 residues. An enhancement of inhibition of $CaCO_3$ crystal nucleation by polyaspartate-polyalanine molecules in solution was observed. Perhaps this enhanced inhibition is due to the disruption of the diffusion of lattice ions to the crystal nuclei by the presence of a hydrophobic layer provided by the polyalanine tails of the molecules.

The hydrophobic regions of matrix molecules could also affect their behavior as promoters of crystallization. In this study, therefore, a polyalanine domain of 8 residues was attached to polyaspartate molecules of 20 or 40 residues. These polyanionic-hydrophobic peptides were then crosslinked to the insoluble chitosan coupons so that the effects on $CaCO_3$ crystallization could be evaluated. Attaching the hydrophobic tail to polyaspartate of 20 residues significantly suppressed promotion of crystallization by the immobilized peptide. However, the polyalanine domain attached to polyaspartate of 40 residues had no measurable influence on the promotion of crystallization by the molecules. This is consistent with the observation of Sikes *et al.* (1990), who reported that polyaspartate molecules of about 15 to 20 residues had the optimal size for interaction with $CaCO_3$ crystal nuclei. Although portions of the molecules were occupied by adsorption to crystal nuclei, polyaspartate molecules of 40 residues seemed sufficiently large so as to occupy the zone of diffusion around the crystal surfaces, with or without the additional polyalanine tail.

In the context of the present studies, the results suggest that the negatively charged residues of the $\text{aspartate}_{20}\text{alanine}_8$ molecules may have been occupied principally with the surface of the crystal nuclei, and the hydrophobic region may have been extruding from the surface, impeding access to the nucleation template. On the other hand, the $\text{aspartate}_{40}\text{alanine}_8$ molecules may have presented a significant number of negatively charged residues that were not associated with the surface of the crystal nuclei, and were thus available as possible sites for interaction with crystal nuclei, regardless of the presence of the polyalanine domain. In any event, the relative sizes of polyanionic and hydrophobic regions can clearly influence the tendency for a peptide or protein to function as a promoter of crystallization, and one possible function of the hydrophobic zone of matrix molecules is to regulate the promotion of crystallization. Other possible effects of the hydrophobic region, such as changes in secondary structure that might favor peptide interactions with crystals, as described by Addadi and coworkers (1985, 1986, 1987, 1990), also need to be evaluated.

Although the immobilized matrix complexes in this study promoted crystallization, crystal growth rates were suppressed relative to control experiments. That is, crystal growth did not occur in the bulk solution when immobilized matrix complexes were suspended in the solution. However, crystal growth (white, cloudy precipitate) did occur in the bulk solutions of controls and experiments with chitosan coupons without crosslinked peptide or protein. Similarly, Bernhardt *et al.* (1985) observed that mineral was induced on the IM framework, and that no crystals were visible in the solution. Typically, mineral induction has been demonstrated by the presence of crystals on immobilized matrix complexes (Greenfield *et al.*, 1984; Bernhardt *et al.*, 1985; Lussi *et al.*, 1988; Roer *et al.*, 1988) rather than in the solutions. Roer *et al.* (1988) demonstrated, by polarized light microscopy, the presence of crystals grown on decalcified pre- and post-ecdysial cuticle after *in vitro* mineralization. In the present study, crystal deposition on the immobilized matrix complex was observed by acid-labile birefringence of a sample after suspension in the bulk solution. Calcium measurements by atomic absorption spectrophotometry, before and after the experiment, also indicated crystal deposition on the coupon in amounts that were consistent with the downward pH shifts that accompanied the removal of carbonate from solution during crystallization. Deposition of crystal on the immobilized matrix complex and inhibition by diffusible inhibitors such as SM may account for the lack of crystal growth in bulk solutions.

Various mechanisms have been proposed to explain nucleation events. For example, the epitaxial hypothesis requiring spatial matching between immobilized anionic groups and the Ca-Ca distance in the lattice suggests specific amino acid sequences that might function as nucleation sites. Therefore, studies involving formation of peptide-chitosan complexes are underway to test possible mechanisms of matrix-crystal interactions.

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Rhizophydium littoreum on the Eggs of *Cancer anthonyi:* Parasite or Saprobe?

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Abstract. The relationship between host and symbiont is often difficult to assess and quantify. A novel technique that may help assess the host-symbiont relationship of organisms found in crab egg masses is described. This technique may have application in determining the relationship of other host-symbiont associations. Crab eggs were killed cryogenically and exposed in combinations with live eggs to a previously unreported symbiont of crab egg masses. The results indicated that the chytrid *Rhizophydium littoreum* is primarily a saprobe that attacks dead eggs; yet at high zoospore densities, it attacks and kills live eggs. Furthermore, *R. littoreum* is the first chytridiomycete to be reported from a marine crustacean host. It was highly prevalent on the eggs of its host and was found throughout the year.

Introduction

Symbionts are, broadly speaking, two organisms living in association together (de Bary, 1879). There is a wide range of relationships that symbioses encompass—*e.g.*, mutualism, commensalism, and parasitism (Noble and Noble, 1985)—and these relationships are often difficult to define. Several symbionts live in the broods of commercially important crabs and lobsters [*e.g.*, *Callinectes sapidus* (Rogers-Talbert, 1948), *Cancer anthonyi* (Shields *et al.*, 1990), *Cancer magister* (Fisher and Wickham, 1976; Wickham, 1986), *Homarus americanus* (Aiken *et al.*, 1985; Campbell and Brattey, 1985), *Paralithodes camtschatica* (Wickham *et al.*, 1985; Kuris *et al.*, 1991)]. Indeed, some of these symbionts are egg parasites or predators that may cause widespread brood losses in certain commercial stocks of crustaceans (Wickham, 1986; Kuris and Wickham, 1987; Kuris *et al.*, 1991). Species of bacteria, zoosporic fungi, nemerteans, and amphipods have been found together on individual crab hosts, and all have been implicated as agents that cause egg mortality. The contributions of these symbionts to egg mortality in populations of some of these decapod hosts have only recently been elucidated (Shields and Kuris, 1988; Kuris *et al.*, 1991).

This is the first report of a chytrid symbiont infesting a marine crustacean host, the yellow rock crab, *Cancer anthonyi*. The fungus-like chytrid, *Rhizophydium littoreum* Amon, 1984, was recovered and isolated from the eggs of *C. anthonyi* during a field survey for the presence of egg mass symbionts (Shields and Kuris, 1988; Shields *et al.*, 1990). The prevalence of the chytrid in the broods of *C. anthonyi* prompted an investigation into its role as a possible agent of egg mortality. The host-symbiont relationship was examined in laboratory studies that included a novel experimental protocol.

Materials and Methods

Ovigerous crabs were trapped in the Santa Barbara Channel, between Summerland and Gaviota, California. The crabs were collected at depths of 10–100 m by a commercial fisherman, transported directly to the laboratory, and maintained at ambient seawater temperatures in 280-l flow-through fiberglass aquaria.

The presence of *R. littoreum* was established as follows. The egg samples were removed to sterile petri dishes containing UV-filtered seawater (2 × 35 μm activated charcoal filters, one ultraviolet-light filter, Rainbow Plastics, Filter Division, El Monte, California) for direct examination

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with a stereo microscope. After three days the samples were observed again and streaked with sterile pipettes onto a sterile modified Vishniac medium (MV): 1.0 g glucose, 1.0 g gelatin hydrolysate, 0.1 g bacto-peptone, 0.1 g yeast extract, 1.0 l seawater, 15.0 g agar (modified from Fuller *et al.*, 1964), containing antibiotics (500 mg each of penicillin-G and streptomycin sulfate per liter). Seawater controls were also cultured. After an additional 3–5 days the plates were examined for the presence or absence of chytrid thalli. Other substrates from the habitat of *C. anthonyi* were not examined for chytrids.

Pure cultures of *R. littoreum* were isolated from the eggs of different crabs on numerous occasions. Isolated cultures of *R. littoreum* were grown in sterile liquid MV medium (MV as above with 1.0 g agar instead of 15.0 g agar). Cultures were maintained both with, and without, antibiotics (500 mg/l each of penicillin-G and streptomycin sulfate, Sigma Co.) at 15° and 20°C.

To establish the host-symbiont relationship, live and dead crab eggs (see below) were exposed to the chytrid separately and in combination. Before exposure, egg-bearing setae were removed from the pleopod and placed in UV-filtered seawater. Samples consisting of 80–300 eggs that were attached to individual and intertwined setae were counted, and the number of dead eggs and their apparent cause of death (*e.g.*, mechanical disruption, infertility, etc.) were noted. After they had been counted, the samples were washed in UV-filtered seawater containing 1.0% bleach for 3–5 min to kill or remove microorganisms. They were then placed in 35 × 10 mm plastic petri dishes with 3.0 ml of UV-filtered seawater containing antibiotics (500 mg/l each of penicillin-G and streptomycin sulfate). Combinations of live and dead eggs (80–300 of each per replicate) were then exposed to approximately 1000 zoospores of *R. littoreum*.

Samples of egg-bearing crab setae (80–300 eggs/sample) were plunged into liquid Freon (Pelco) in a metal dish jacketed with liquid nitrogen. The eggs thus killed were thawed in ice cold seawater, and equilibrated to 15°C. The samples were then counted and the few broken eggs were recorded. The coats of the eggs killed in this manner were not grossly disrupted.

The effect of zoospore density on mortality was determined by exposure of eggs to 10, 100, and 1000 zoospores/ml at 15°C. Zoospores from cultures of *R. littoreum* were counted with the aid of a hemocytometer (Levy counting chamber). Three replicates of the culture were counted and the appropriate dilutions were made to give estimated densities of 10, 100, and 1000 zoospores/ml. From eight to ten replicates were examined in each treatment. A separate treatment of eggs exposed to antibiotics and diluted MV medium served as the control. Crab egg mortality (*i.e.*, the number of living or dead eggs attacked by the chytrid) was assessed every two to three days for ten days. Eggs in control exposures experienced negligible mortality.

Results

Rhizophydium littoreum was identified by Dr. D. J. Barr (Fig. 1). Representative specimens (Barr #580) have been deposited at the Biosystematics Research Centre (Wm. Saunders Bldg., C.E.F. Ottawa, Ontario, K1A 0C6, Canada). The monocentric thalli of *R. littoreum* ranged from 30 to 90 μm wide on crab eggs and in MV medium. Smaller immature thalli were also observed. The thallus was epibiotic and typically resided externally on the crab egg with the rhizoids penetrating through the egg coat into the contents of the egg. An apophysis was occasionally observed in MV culture. In culture, the life cycle of the chytrid took approximately 3–5 days from the zoospore stage to the production of a mature sporangium (at 15°C).

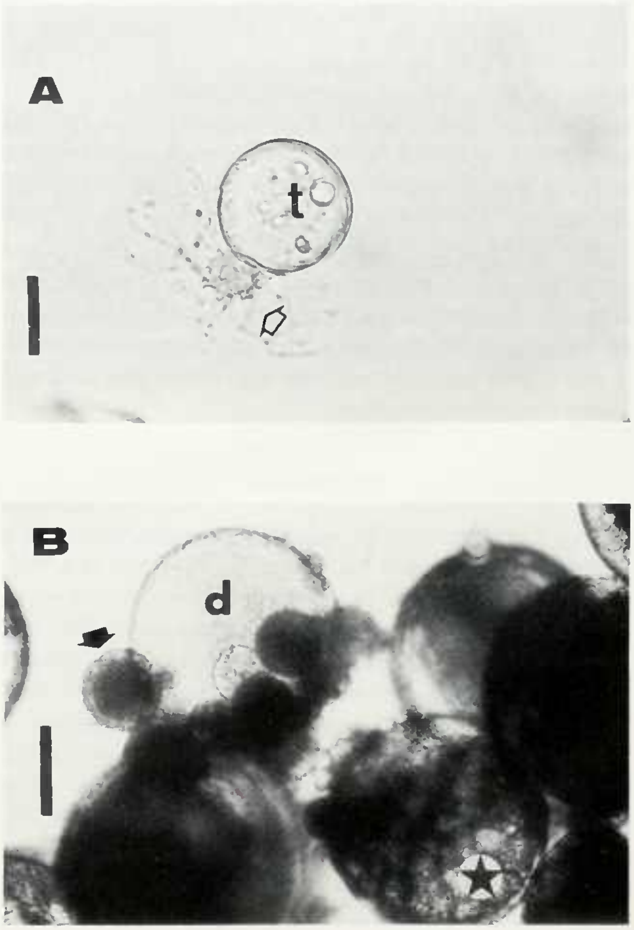

Figure 1. (A) *Rhizophydium littoreum* from culture; sporangium (t) and rhizoids (arrow); bar = 50 μm. (B) *Rhizophydium littoreum* in live eggs only treatment. Note the dead egg (d) with thallus attached (arrow), and live egg (star); bar = 100 μm.

Zoospores were posteriorly uniflagellate and ranged in size from 3 to 4 μm in diameter.

Live eggs that were successfully attacked by the chytrid lost all internal integrity over the course of 2 days at 15°C. Live eggs that were unsuccessfully attacked by the chytrid developed normally. The chytrid survived infrequently on the external coat of unsuccessfully attacked eggs. The thalli of these chytrids did not grow larger than 40 μm and did not produce zoospores. The rhizoids of these thalli were directed outward into the surrounding medium and did not appear to penetrate the egg coat.

The prevalence of *R. littoreum* from *Cancer anthonyi* was established by regular monthly or bimonthly samples of eggs taken from crabs from the Santa Barbara Channel off the coast of southern California. Prevalence ranged from 14 to 52% of the broods examined (overall prevalence = 29%, $N = 225$) throughout the year (Oct. 1985–Sept. 1986) but showed no significant seasonality ($G_H = 12$, $P < 0.10$, $df = 8$).

Rhizophydium littoreum attacked dead eggs preferentially (Figs. 2, 3). No significant differences were observed between the proportion of dead eggs attacked by the chytrid in the presence or absence of live eggs (80–300 of both live and dead eggs) (*t*-test between dead with live and dead only, $P < 0.05$, 7 of 7 comparisons at day 10). In the presence of live and dead eggs, the chytrid preferred dead eggs. Significantly more dead eggs were attacked by the chytrid than were live eggs (ANOVA, Bonferroni's inequality, $P < 0.05$, four comparisons/experiment), except in the EDS I treatments between live eggs and dead eggs exposed to 100 zoospores (Fig. 3B, C). It attacked and killed live eggs only at high zoospore densities (Fig. 2, 1000 zsp/ml initial exposure) or when zoospores were visible at high densities in treatments (Fig. 3A, B, typically 3–6 days after exposure). In the presence of dead eggs, significantly fewer live eggs were attacked and killed in several treatments than were live eggs from separate exposures (*t*-test between live with dead and live only, $P < 0.05$, 3 of 7 comparisons at day 10). No significant differences were observed in the proportion of live eggs attacked by the chytrid in 4 of 7 comparisons (at day 10) in the presence or absence of dead eggs (*t*-test as above, $P > 0.05$).

The stage of egg development did not influence the proportion of dead eggs attacked by the chytrid. In contrast, on live eggs, the chytrid preferred eggs in later stages of embryogenesis (Fig. 2). Significantly fewer live eggs were attacked in early stages of embryogenesis (EDS I) than in later stages (ANOVA, arcsin transformation of proportions, Sidak's inequality, $P < 0.01$), but this pattern was not consistent between experiments (*e.g.*, compare Fig. 3B, E).

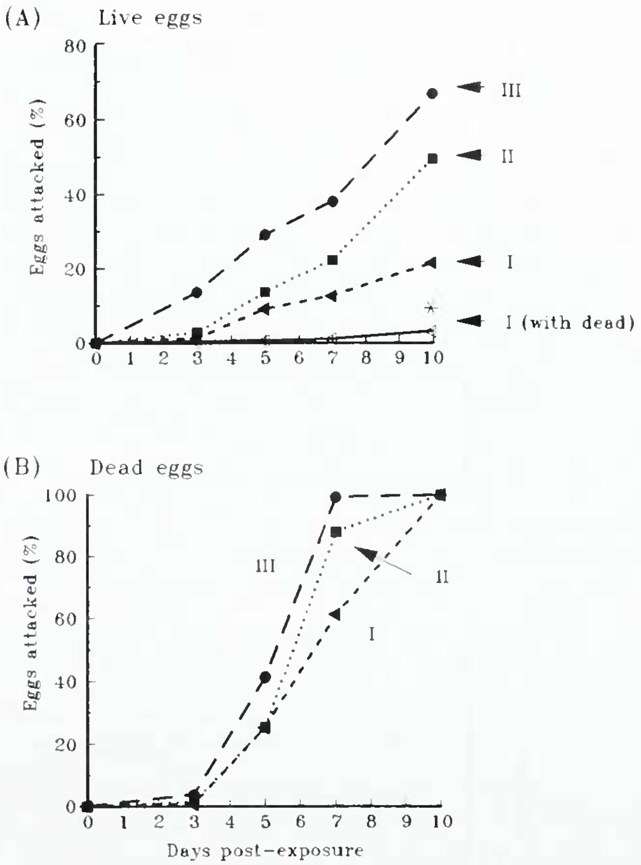

Figure 2. Infection dynamics of *Rhizophydium littoreum* on (A) live eggs and (B) dead eggs after exposure to 1000 zoospores. Live eggs in the presence of dead eggs are noted (with dead). Roman numerals refer to early (EDS I), middle (EDS II), and late (EDS III) stages in embryogenesis (Shields and Kuris, 1988; Shields *et al.*, 1990). Error bars not shown. *$P < 0.05$, significantly different from treatment with live eggs alone (*t*-test between EDS I live eggs alone and live eggs with dead eggs, at day 10). Not shown is the dead eggs in the presence of live eggs treatment. The data were not significantly different from the dead eggs only treatment.

Discussion

The results show conclusively that *R. littoreum* can kill live eggs, but it prefers dead eggs. Under natural conditions (*i.e.*, low zoospore density), *R. littoreum* may be a facultative parasite, but it is more likely a saprobe that lives on dead eggs. Indeed, in some cases, fewer live eggs were attacked by the chytrid when dead eggs were present than when dead eggs were absent.

Less conclusive in the laboratory was the relationship between crab embryogenesis and chytrid-related mortality. Consistent patterns were not observed (*e.g.*, Fig. 3B, E). Variations in fungal pathogenicity or host resistance/susceptibility manifested by slower growth rates of the thalli or decreased zoospore production may account for

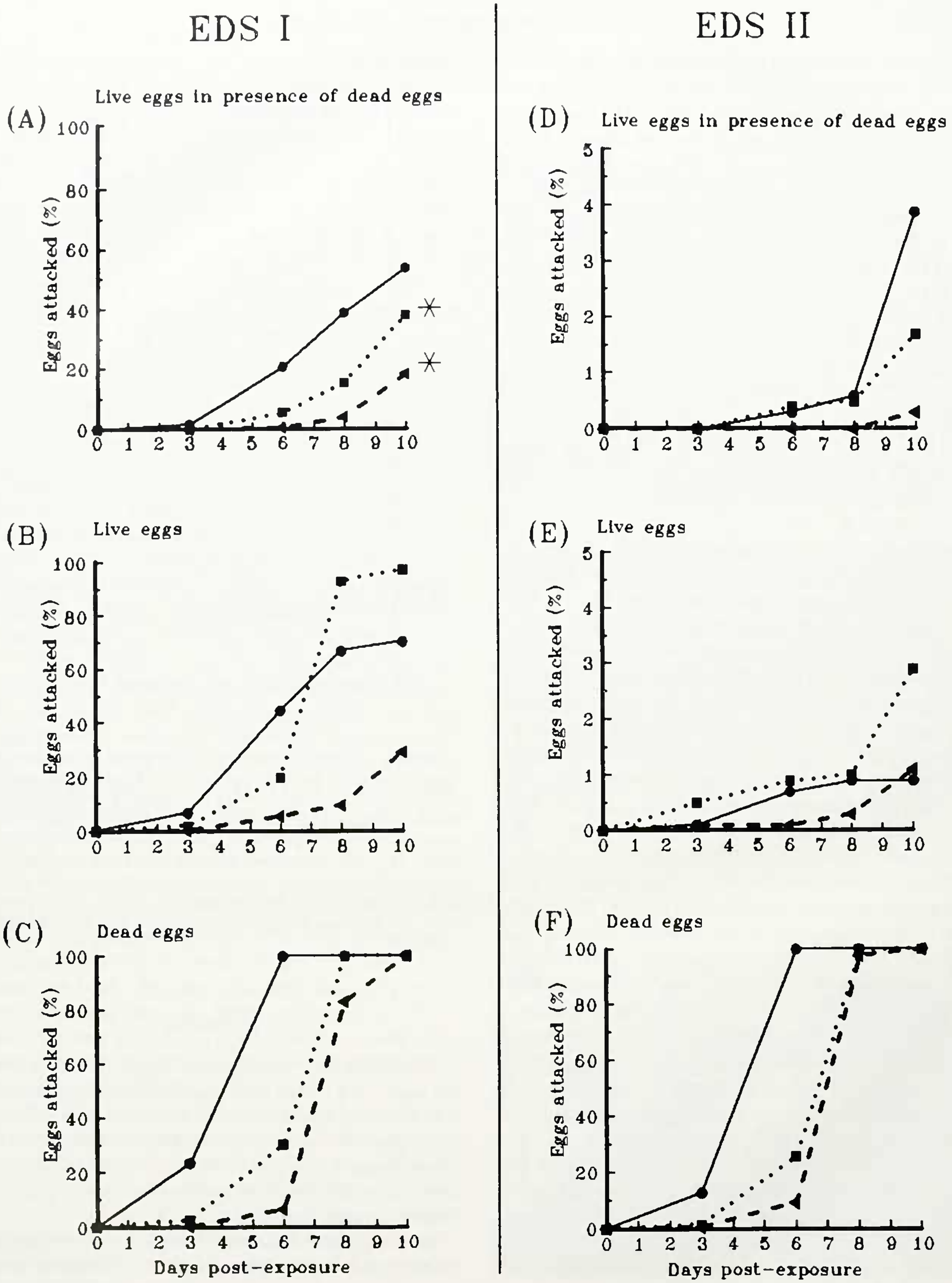

Figure 3. Infection dynamics of *Rhizophydium littoreum* on eggs of different developmental stages (EDS I or II) after exposure to different zoospore densities. Eggs were exposed to densities of 10 zoospores (triangles and dashed lines), 100 zoospores (boxes and dotted lines), and 1000 zoospores (circles and solid lines). Error bars not shown. $^*P < 0.05$, significantly different from treatments with live eggs alone (*t*-test, day 10). Not shown is the dead eggs in the presence of live eggs treatment. The data were not significantly different from the dead eggs only treatment.

the differences in attack rates of the chytrid between EDS classes. Pathogenicity and resistance have been examined in fungi-plant associations (*e.g.*, Miedaner, 1988; De Nooij and Van Damme, 1988; Alexander, 1989), but have received scant attention in marine associations. The experimental protocol can easily be manipulated to examine these factors in more detail.

Chytridiomycetes develop in a variety of live and dead fungi, algae, diatoms, and higher plants (Sparrow, 1963). They damage host cells or tissues by direct penetration of rhizoids, which in the living host results in death. Chytrids can occur as saprobes, and facultative and obligate parasites in nature (Barr, pers. comm.).

Previously, *Rhizophydium littoreum* has only been reported from the siphonous green algae, *Bryopsis plumosa* and *Codium* sp. (Kazama, 1972; Amon, 1984). It can be readily established in different culture media (Kazama, 1972; Amon, 1984, 1986). Chytrid parasites of other Metazoa have been reported from the eggs of cestodes and rotifers (Sparrow, 1963) and from aquatic copepods and ostracods (Whisler *et al.*, 1974; Weiser, 1977). A chytrid-like organism from the branchial lamellae of a shrimp (Uzmann and Haynes, 1969) may be a thraustochytrid, *Schizochytrium*.

Dead eggs are abundant in the broods of many decapod crustaceans (for review see Kuris, 1991). Indeed, populations of several commercially harvested species have recently suffered catastrophic brood losses to symbiotic agents (Wickham, 1986; Kuris *et al.*, 1991). *Cancer anthonyi*, however, experiences a relatively small degree of egg mortality (~5.0%, Shields *et al.*, 1990). This small degree of egg mortality translates into several thousand dead eggs per brood because a large (>140 mm carapace width) *Cancer anthonyi* can oviposit up to 3 million eggs (Shields, 1991). Hence, sufficient dead eggs to provide a substrate for *R. littoreum* may occur in the egg masses of the majority of the ovigerous population of *C. anthonyi*. In addition, female *Cancer* crabs bury themselves in the substrate during oviposition; their broods may become infested with the chytrid at that time.

Several species of zoosporic fungi occur on the eggs of decapods (*e.g.*, *Atkinsiella dubia*, *Haliphthoros milfordensis*, *Lagenidium callinectes*, *Leptolegniella marina*, *Pythium thalassium*). The use of live and dead egg treatments may help to establish the role of these zoosporic fungal "pathogens." In addition, the experimental protocol may help to elucidate the roles of egg parasites in other systems.

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Collagen in the Spicule Organic Matrix of the Gorgonian *Leptogorgia virgulata*

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Abstract. Decalcification of the calcareous spicules from the gorgonian *Leptogorgia virgulata* reveals an organic matrix that may be divided into water insoluble and soluble fractions. The insoluble fraction displays characteristics typical of collagen, which is an unusual component of an invertebrate calcium carbonate structure. This matrix fraction exhibits a collagenous amino acid profile and behavior upon SDS-PAGE. Furthermore, the reducible crosslink, dihydroxylysinonorleucine (DHLNL), is detected in this fraction. The composition of the matrix varies seasonally; *i.e.*, the collagenous composition is most prevalent in the summer. These results indicate that the insoluble matrix is a dynamic structure. Potential roles of this matrix in spicule calcification are discussed.

Introduction

The mesoglea of the gorgonian *Leptogorgia virgulata* contains microscopic calcite (calcium carbonate) spicules (Kingsley and Watabe, 1982). Isolation and decalcification of the spicules yield an organic matrix, which is intimately involved in calcification (see Wilbur and Simkiss, 1968; Weiner and Traub, 1981; Watabe, 1981).

Unlike vertebrate osseous tissues that consist of hydroxyapatite (calcium phosphate) and collagen, collagen has not been associated with the formation of invertebrate calcium carbonate structures (Jope, 1967; Watabe, 1981; Benson *et al.*, 1983; Swift *et al.*, 1986). The presence of collagen, or a collagen-like component, in the spicule proteins of gorgonians had been suggested previously (Silberberg *et al.*, 1972; Goldberg, 1988), but this could not be confirmed (Kingsley and Watabe, 1983).

This report presents conclusive evidence for a predominant, insoluble "classic" collagen component within isolated and apparently homogeneous calcite spicules of *L. virgulata* collected in the summer months. The collagen is partially characterized, and noncollagenous components of the insoluble matrix are examined as well. Potential roles of the insoluble matrix in spicule calcification are discussed.

Materials and Methods

Colonies of the gorgonian *Leptogorgia virgulata* were collected at low tide from Sixty Bass Creek of North Inlet Estuary, Georgetown, South Carolina, in the summer of 1985 and from the subtidal waters off Morehead City, North Carolina, in March, July, and December of 1987. Colonies were immediately cleaned of adhering organisms and debris, frozen, and transported on dry ice and stored at −30°C.

Organic matrix preparation

All preparations were conducted at 4°C unless otherwise indicated. The tissues of the colonies that contain the spicules were stripped from their axes, weighed, and washed with 0.02 M NH_4HCO_3. The tissue was suspended in 10 volumes of 0.25 M NaCl in 0.2 M NH_4HCO_3 buffer adjusted to pH 8.0. The spicules remained insoluble under these conditions. They were released from their surrounding tissues by digestion (24 h, at 37°C, with shaking)

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff's reagent; TES, N-Tris-(hydroxymethyl) methyl-2-aminoethane sulfonic acid; DHLNL, dihydroxylysinonorleucine.

with 1% papain (substrate/enzyme, w/w) activated with 0.005 *M* cysteine. The supernatant was decanted, and any undigested tissue was treated with additional papain, as described above, for another 24 h. The tissues surrounding the spicules were completely digested following these treatments. The remaining spicules were washed thoroughly with the above buffer and retained on a 250 μm mesh sieve. The spicules, while contained in the sieve, were washed thoroughly with the same buffer to avoid any possible contamination by enzyme or solubilized material. Examination under a microscope indicated an apparently homogeneous preparation of spicules.

Spicules were suspended in an equal volume of 0.5 *M* potassium EDTA in 0.05 *M* NH_4HCO_3, pH 8.0, and demineralized by dialysis (with 3500 dalton cut off tubing) against the same solution. Following demineralization, the total content was recovered from the dialysis tubing and centrifuged. The insoluble organic matrix residue was washed 3 times with 0.2 *M* NH_4HCO_3, 6 times with 0.05 *M* NH_4HCO_3, and 3 times with distilled water. The washed insoluble matrix was lyophilized. The supernatant and the washings, which contained the soluble matrix proteins, were dialyzed exhaustively against distilled water (same cut off tubing), and then lyophilized.

Amino acid analysis

The amino acid compositions of: (1) total insoluble matrices from South Carolina, and March, July and December samples from North Carolina; (2) subfractions of the North Carolina July insoluble matrix (described below); and (3) the soluble matrices from July and December were determined. Each sample was hydrolyzed in 200 μl of 6 *N* HCl in an N_2 atmosphere, for 20 h at 110°C. Amino acid analysis was performed on a Varian 5560 Liquid Chromatograph using a stainless steel cation-exchange column (0.4 × 25 cm, AA 911, Interaction) with post column ninhydrin detection. Color was developed at 135°C in a stainless steel reaction coil (Yamauchi *et al.*, 1986).

Collagen characterization

The insoluble matrix was treated with 5% pepsin (w/w) in 10 volumes of 0.5 *M* acetic acid for three days at 21°C. The reaction mixture was centrifuged at 25,000 RPM in an ultracentrifuge for 1 h. The insoluble portion was washed thoroughly with 0.5 *M* acetic acid and lyophilized. The supernatant was brought to 3.0 *M* NaCl in the cold and allowed to stand overnight. The precipitate, after centrifugation at 10,000 RPM for 20 min, was redissolved in 0.1 *M* acetic acid, exhaustively dialyzed against 0.1 *M* acetic acid, and lyophilized. The supernatant of the 3.0 *M* NaCl solution was dialyzed exhaustively against distilled water, and lyophilized. No material was observed. The original insoluble matrix, pepsin solubilized material and pepsin-insoluble material were hydrolyzed as described above and subjected to amino acid analysis.

Cyanogen bromide (CNBr) cleavage

The various fractions obtained above were treated with 25% mercaptoethanol in 0.2 *M* NH_4HCO_3 at 55°C overnight to completely reduce any methionine sulfoxide residues back to Met (methionine). These fractions were then lyophilized, digested with CNBr in 70% formic acid in an N_2 atmosphere for 4 h at room temperature, diluted with distilled water, and lyophilized again.

Polyacrylamide gel electrophoresis (PAGE)

Portions of each fraction, both treated and untreated with CNBr, were subjected to PAGE in 0.1% SDS (Laemmli and Favre, 1973). Soluble type I collagen from foetal bovine skin was used as a standard. Tube gels (4% acrylamide) and gradient slab gels (3–17% acrylamide) were employed. Some of the very insoluble samples were treated at 100°C for 10 min with 0.2% SDS electrode buffer (Laemmli and Favre, 1973) to which 2 *M* urea was added. Gels were stained for protein with 0.05% Coomassie Brilliant Blue, and for carbohydrates with the PAS reagent (Zacharius *et al.*, 1969).

Determination of cross-links

Samples of lyophilized untreated insoluble spicule matrix were suspended in 0.15 *M* TES buffer, pH 7.5, and reduced with standardized NaB^3H_4 (Fukae and Mechanic 1980; Yamauchi *et al.*, 1986). The reduced insoluble matrix was hydrolyzed *in vacuo* with 3 *N* HCl for 48 h at 115°C (Yamauchi *et al.*, 1986). Analysis of cross-links was performed on a Varian 5560 Liquid Chomatograph as described previously (Yamauchi *et al.*, 1986). Hydroxyproline (Hyp) analysis was performed by amino acid analysis and residues of cross-link per mole of collagen was calculated on the basis of 300 residues of Hyp per molecule collagen.

Results

Amino acid compositions

The amino acid compositions of the matrix fractions of gorgonians collected from South Carolina and North Carolina are shown in Table I. Also presented are amino acid compositions of an invertebrate collagen and a mammalian mineralized collagen (Table I I, J, respectively). The compositions of the insoluble matrices of the two summer samples are similar (Table I A, C). Both samples display compositions typical of a collagen, since they contain significant amounts of Hyp, Hyl (hydroxy-

TABLE I

Amino acid compositions of matrix protein fractions from isolated calcite spicules of Leptogorgia virgulata

	Residues per 1000 Total Residues									
	A TIM S.C.	*B* TIM 3/87	*C* TIM 7/87	*D* TIM 12/87	*E* Pepsin sol	*F* Pepsin res	*G* SM 12/87	*H* SM 7/87	*I* Sea anemone collagen^d	*J* Bovine bone^e
Hyp	74	8	82	31	80	11	1	—	103	98
Asp	93	132	76	130	84	153	516	577	73	45
Thr^a	36	57	34	48	32	63	18	16	37	17
Ser^a	43	57	42	53	43	78	16	13	41	34
Glu	109	87	94	92	112	76	34	32	97	74
Pro	66	67	65	59	64	50	17	19	67	123
Gly	295	158	332	225	335	161	210	185	339	337
Ala	83	78	82	76	77	67	130	108	65	109
Val	27	58	26	37	22	55	17	16	25	20
Cys^b/2	2	16	1	10	—	17	—	—	—	—
Met^c	4	11	7	10	5	10	—	—	—	5
Ile	18	38	15	26	13	46	6	5	22	11
Leu	27	53	26	42	23	58	7	6	30	25
Tyr	8	24	8	20	6	32	—	2	3	4
Phe	9	31	9	22	7	35	4	4	8	13
His	3	17	4	11	3	11	2	1	1	4
Hyl	33	7	34	15	34	6	—	—	25	6
Lys	15	44	12	33	10	33	12	9	16	26
Arg	56	55	50	53	50	39	8	5	68	50

TIM—Total Insoluble Matrix.
S.C.—Summer collection in South Carolina.
3/87, 7/87, 12/87—Dates of collection in North Carolina.
Pepsin sol—7/87 collection, pepsin soluble material.
Pepsin res—7/87 collection, pepsin insoluble material.
SM—Soluble Matrix.
[a] Uncorrected for hydrolysis.
[b] Half Cys, sum of cysteic acid and cystine.
[c] Sum of methionine sulphoxide and methionine.
[d] Nowack and Nordwig 1974.
[e] Herring 1972.

lysine) and 33% Gly (glycine). The composition in column C is extremely close to that of the pepsin solubilized invertebrate collagen of the sea anemone (column I). The amino acid composition of the insoluble matrix from samples collected in March 1987 displays markedly lower values of Hyp, Hyl and Gly (Table IB). Gly, Asp (aspartic acid) and Glu (glutamic acid) are the most abundant amino acids. The insoluble matrix from samples collected in December 1987 displays an amino acid composition that is intermediate to the March and July compositions (Table ID).

The July insoluble spicule matrix, which was partially solubilized by pepsin digestion in 0.5 *M* acetic acid and precipitated by addition of 3.0 *M* NaCl, also has the composition typical of a collagen (Table IE). This precipitated collagen is a white fibrous material that displays an amino acid composition similar to that of the whole insoluble matrix (Table IC) and sea anemone collagen (Table II). The amino acid composition of the fraction of the July insoluble matrix that is not soluble in pepsin and acetic acid (Table IF) is similar to that of the insoluble matrix of the spicules collected in March (Table IB).

The soluble matrix fractions are off-white and extremely hygroscopic. Samples from December and July display similar amino acid compositions. The most prominent feature of this fraction is its extremely high aspartic acid content (>50%).

PAGE

The pepsin solubilized spicule collagen was subjected to 4% acrylamide SDS-PAGE and compared to type I bovine soluble skin collagen (Fig. 1). Although the bands of the spicule matrix are faint, they can be seen to travel in the range of type I collagen. The spicule collagen contains a component similar in mobility to the $\alpha 1$ (I) chain.

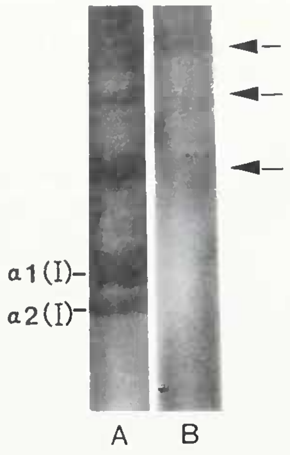

Figure 1. SDS-PAGE of (A) type I collagen, and (B) the total insoluble matrix of *Leptogorgia virgulata*.

Also present are molecular weight components equal to the β and γ chains of type I collagen.

A CNBr digest of the solubilized collagen from the July insoluble matrix showed, on 3–17% acrylamide gradient gels, different patterns from that of a CNBr digest of type I collagen (Fig. 2). Treatment with urea did not change the pattern of the insoluble matrix. The total insoluble matrix apparently did not enter the gel and therefore remained uncleaved by CNBr.

SDS-PAGE of the matrix not solubilized with pepsin revealed low molecular weight proteins that stained with PAS (Fig. 3), indicating the presence of glycoproteins in this matrix.

Following the NaB^3H_4 reduction of the insoluble matrix, cross-link analysis indicated that the only reduced cross-link present was DHLNL (Fig. 4). Each nmole of collagen contained 2.87 nmoles of DHLNL. No stable non-reducible cross-links were detected.

Discussion

This report is the first to demonstrate conclusively the presence of a "classic" collagen (*i.e.*, a protein containing Hyp, Hyl, and 33% Gly as well as a typical collagen cross-link) as part of the organic matrix of a calcite (calcium carbonate) invertebrate skeletal structure. The insoluble organic matrices from calcite spicules of *Leptogorgia virgulata* collected in the summer months have amino acid compositions characteristic of collagen (Table IA, C). Those compositions are similar to other soft tissue coelenterate collagens (Franc, 1985). When the amino acid composition is viewed *in toto*, strong similarities to the pepsin soluble fraction, and sea anemone and vertebrate collagen are evident (see Table I C, E, I, J). The SDS-PAGE pattern of the July insoluble matrix (after pepsin digestion) also displayed the characteristic of type I collagen (100,000–300,000 daltons). However, the *Leptogorgia* matrices do not exhibit periodic bandings of typical vertebrate collagens (Watabe and Kingsley, unpub.).

The intermolecular reducible cross-link, dihydroxylysinonorleucine (DHLNL), was clearly detected in the collagen of the July insoluble matrix. Little if any other reducible and non-reducible cross-links were present. Similar observations have been made in other invertebrate collagens (Shadwick, 1985) including coelenterates (Bailey, 1971). The latter were from non-mineralized tissues. DHLNL is the most prevalent reducible cross-link in the type I collagen of bovine tendon, bone, and dentin (Mechanic *et al.*, 1971). Mechanic *et al.* (1985) have demonstrated that DHLNL is distributed in different molecular locations in these three tissues and may determine the functional properties of collagen. They suggest that the presence of multifunctional cross-links (*i.e.*, histidinohydroxylysinonorleucine, pyridinoline, and histidinohydroxymerodesmosine) in the nonmineralized collagen tissues holds the molecules at a shorter distance than do the bifunctional cross-links of the mineralized collagen in bone, thereby physically precluding the entrance of ions and the subsequent formation of hydroxyapatite crystals. Conversely, once bone collagen is calcified, the mineral does not allow close enough juxtaposition of the molecules to form multifunctional cross-links (Mechanic *et al.*, 1985). The molecular location of DHLNL is not known in the spicule matrix of gorgonians, however, consistent with this theory, multifunctional cross-links are not present.

We have used enzymic digestion by papain at pH 8.0 at 37°C in order to isolate a homogeneous population of calcite spicules. The mineral contained in the collagen of a mineralized tissue protects collagen from denaturation, as well as from enzymatic degradation at neutral pH and

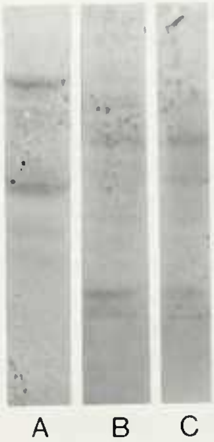

Figure 2. Cyanogen bromide cleavage patterns of (A) type I collagen; (B) pepsin solubilized insoluble matrix; and (C) pepsin solubilized insoluble matrix treated with urea.

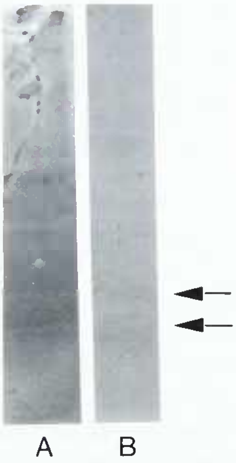

Figure 3. SDS-PAGE of the fraction of the insoluble matrix not solubilized in acetic acid and pepsin, stained for (A) protein with Commassie blue, and (B) carbohydrate with PAS.

above (Bonar and Glimcher, 1970). The physiological, relatively gentle digestion procedure used in this study degraded and solubilized all the collagenous and non-collagenous protein that was not protected by mineral. Previous methods used to isolate "calcified" structures used NaOCl or a strong base to destroy organic material. However, NaOCl will produce anorganic bone from normal mammalian bone and is often used to examine bone architecture by scanning electron microscopy. This also degrades as well as destroys a significant portion of the organic matrix of mineralized tissue. The above analysis is substantiated by a comparison of the current results with a previous failure to detect collagen in the insoluble matrix fraction (Kingsley and Watabe, 1983). Kingsley and Watabe (1983) isolated spicules in 5.25% NaOCl and demineralized in 0.1 *N* HCl at room temperature. The isolated spicules that were analyzed were, in both cases, from animals collected during the summer, so the previous methods were harsh and yielded erroneous results.

The spicule insoluble matrix of the gorgonian *Briareum asbestinum* consisted of very small peptides (1600–5000 daltons) and contained 22 Hyp residues/1000, approximately 20% Gly and no Hyl (Silberberg *et al.*, 1972). These features only suggest the presence of collagen-like components. The amino acid composition of the whole spicule matrix of the gorgonian *Pseudoplexaura flagellosa* revealed 48, 237, and 9 residues/1000 total residues of Hyp, Gly, and Hyl, respectively (Goldberg, 1988). Silberberg *et al.* (1972) air dried, ground the whole animals, digested the organic material with 30% KOH, and demineralized in concentrated HCl at 4°C. These procedures are extremely severe in the processing of biological material and may have resulted in the loss of collagenous components. The isolation of spicules in 1 *N* NaOH by Goldberg (1988) may have caused similar problems. However, such differences in the matrix compositions of the gorgonian species may also be the results of other factors including species variations, and the season and the environment from which specimens were collected (see below).

The results of the present study indicate that the amount of collagen in the insoluble matrix is directly related to the season of collection and, therefore, to temperature or other environmental conditions. The matrices isolated from animals collected in the summer from both South Carolina and North Carolina display Hyp and Gly levels similar to those of vertebrate collagen, as well as large amounts of Hyl (Table 1 A, C). Matrices from animals collected in March and December are different from the above; *i.e.*, they are less collagen-like in composition. In December, both the Hyp and Hyl contents were less than half of those of summer samples, and the Gly was reduced to 225 residues/1000. By March, the Hyp, Hyl, and Gly contents continued their reduction to 8, 7, and 158 residues/1000, respectively. These results clearly indicate a seasonal variation in the insoluble matrix. Had the analyses of the organic matrix compositions only been conducted on specimens collected in March, the predominance of the collagenous component of the matrix might

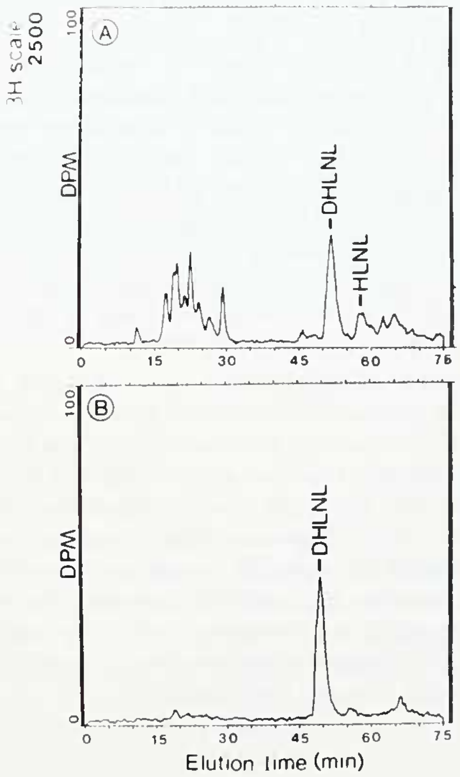

Figure 4. Elution patterns of reduced collagen from (A) bovine bone and (B) *Leptogorgia virgulata* spicule insoluble matrix. The positions of the intermolecular cross-links dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) are shown.

have been overlooked. The seasonal changes in the insoluble matrix are not simply shifts of amino acid residues from insoluble to soluble fractions, because the soluble fractions in summer and winter have relatively similar amino acid compositions (Table I G, H).

The apparent seasonal variation suggests a remodeling or turnover of the collagen component, and thus a degree of demineralization and remineralization (turnover) in the spicules. A comparison of the amino acid composition of the non-solubilized portion of the insoluble matrix and the total insoluble matrix collected in March reveals strong similarities. If indeed there is partial demineralization of spicules in winter months, and the portion of the collagen that is pepsin soluble is lost, then the amino acid composition of the winter matrix and that of the non-solubilized fraction of the summer matrix should be similar. Certainly, in the remodeling of vertebrate bone, demineralization must occur prior to degradation of collagen. Spicules in *L. virgulata* are initially formed intracellularly, but later, they become exposed to the extracellular environment (Kingsley and Watabe, 1982). It is not clear whether spicule growth and maturation continues once these structures are in the extracellular environment, although this apparently occurs in other gorgonian species (Goldberg and Benayahu, 1987). The present results indicate that the spicules are dynamic structures even after they emerge from the cell.

The pepsin solubilized insoluble matrix shows a collagenous composition similar to total insoluble matrix. However, CNBr digestion of this solubilized collagen produced peptide patterns distinct from bovine skin type I collagen. Samples of the total insoluble matrix treated with CNBr remained insoluble even after reduction of any methionine sulphoxide to Met with 25% mercaptoethanol. No material was found to enter the gel. It is obvious that another insoluble protein is present that protects the collagen from digestion by CNBr.

The fraction of the insoluble matrix that was not solubilized by pepsin did not display a typical collagenous amino acid composition. Because it did contain some Hyp and Hyl, however, a portion of the collagen in the matrix is probably blocked from enzymic digestion. This predominantly non-collagenous matrix fraction on SDS-PAGE revealed low molecular weight glycoproteins, as is seen by Coomassie Blue and PAS staining. The presence of carbohydrates in invertebrate calcifying matrices is common and has been described previously in gorgonians (Kingsley and Watabe, 1983; Goldberg, 1988). The possibility that glycoproteins play a role in calcification has been proposed for several organisms, (see Crenshaw, 1972; deJong *et al.*, 1976; Fichtinger-Schepman *et al.*, 1979; Marsh and Sass, 1984).

The roles of collagen in biomineralization have been examined extensively in bones and teeth and may now be extended to the invertebrates. Some of the major theories of how collagen may be involved in the regulation of calcification involve its association with other non-collagenous components such as phosphate and osteonectin (see Prockop and Williams, 1982). Similarly, in *L. virgulata* the structure-functional relationship between the non-collagenous matrix and the insoluble matrix, may determine how the total organic matrix regulates spicule formation. Both Kingsley and Watabe (1983) and the present report indicate that the soluble spicule matrix of *L. virgulata* has a soluble acidic protein containing more than 50% Asp. Much of the calcium binding capacity of invertebrate mineralizing matrices has been attributed to the soluble fraction (see Watabe and Kingsley, 1989).

Although this is the first conclusive report of the presence of collagen within the organic matrix of a calcium carbonate skeletal structure, collagen has been found to be associated, indirectly, with a number of calcium carbonate structures. In the echinoderms, collagen is not found within the sea urchin larval spicule matrix (Blankenship and Benson, 1984; Wilt *et al.*, 1985); however, collagen metabolism is critical for normal spicule formation. Collagen is apparently necessary for providing a permissive substratum in which spicule formation may occur (Blankenship and Benson, 1984). Similarly in the pennatulids, another coelenterate, collagen is closely associated with the calcitic crystals but is not responsible for the nucleation of the mineral (Ledger and Franc, 1978). Other such indirect roles of collagen in invertebrate calcification are discussed by Watabe and Dunkelberger (1979) and Kingsley (1984).

Acknowledgments

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Functional Autonomy of Land and Sea Orientation Systems in Sea Turtle Hatchlings

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Sea turtle hatchlings emerge from underground nests on oceanic beaches and immediately confront two separate problems in orientation. First they must locate the ocean and crawl to it; then they must orient offshore while they swim out to sea in a migration lasting several days.

Visual cues guide hatchlings from the nest to the sea (1, 2), but little is known about the cues used by turtles in the ocean. Nevertheless, the crawl across the beach has long been considered essential to swimming orientation because hatchlings released offshore without a crawl reportedly fail to orient seaward (3, 4). Here we report that hatchling leatherback (*Dermochelys coriacea*) and green (*Chelonia mydas*) sea turtles released offshore consistently swam toward approaching waves and oceanic swells. Wave tank experiments confirmed that swimming hatchlings oriented into waves. A crawl across the beach was not a prerequisite for wave orientation in either the field or lab, indicating that hatchling sea turtles possess two separate orientation systems, each based on different sensory cues and capable of functioning autonomously. The first guides hatchlings on land to the sea; the second, based on wave detection, functions during the ocean migration.

In five field experiments with green turtles and five others with leatherbacks, we monitored the swimming orientation of hatchlings released at various distances offshore near Fort Pierce, Florida. A total of 45 green turtle and 48 leatherback hatchlings were tested. All experiments were conducted between July and September in 1988 and 1989.

Hatchlings were obtained from nests deposited on beaches in the Fort Pierce area. Nests were checked daily until a depression formed above the eggs (indicating the eggs had hatched and an emergence would probably occur that evening). We then carefully dug in the sand and removed hatchlings, placed them into styrofoam boxes, and transported them by motorboat to testing sites 2.0–30.0 km offshore. All turtles were tested and released within 48 h of capture.

Each turtle was placed into a nylon-lycra harness (5) tied by a short line to the side of a spherical, half-submerged floating buoy (Fig. 1A). The buoy was attached by another line to the submerged center of a floating cage (Fig. 1A). Swimming hatchlings exerted sufficient force to easily rotate the buoy. Markings on the buoy were clearly visible from the boat, enabling observers to determine the orientation of the buoy (and thus of the turtle) as the hatchling swam in place. Previous reports have indicated that migrating hatchlings do not change course or alter their behavior in response to nearby boats (3, 5, 6). In the present experiments, driving the boat around the cage at distances of 10–20 m also had no detectable effect on hatchling orientation.

When released into the cage, harnessed hatchlings often dove or circled for their first few minutes in the water. After 2–5 min, nearly every hatchling established an essentially constant swimming course from which it only occasionally deviated. Once a turtle settled on its course (or 6 min elapsed), its orientation was determined once each minute for five minutes with a sighting compass. These five readings were used to calculate the mean angle and vector length for each hatchling, following standard procedures for circular statistics (7).

Hatchlings consistently oriented into approaching waves in all experiments, regardless of distance from shore. Data from two different green turtle experiments, in which waves approached from nearly opposite directions, are

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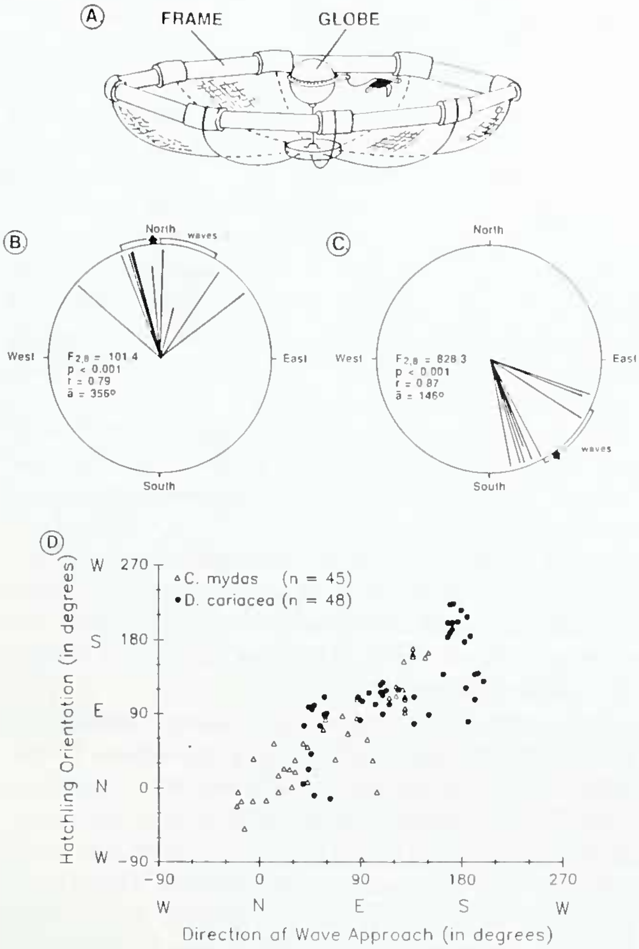

Figure 1. *Results of field experiments with the floating cage (A) The floating orientation cage, globe, and tethered turtle The cage was constructed of eight equal lengths of PVC pipe (5 cm diameter) fused end to end to form an octagonal frame 1.12 m in diameter The frame floated in the water so that only about 1 cm of its upper surface was exposed Stainless steel supports converged on a baseplate 25 cm below the water The hatchling was tethered to a buoyant globe anchored to the baseplate by a thin, nylon line. The turtle could thus swim in any direction, while markings on the globe enabled observers in a nearby boat to monitor its swimming orientation The bottom of the cage was encased in a mesh net to exclude predators A detailed description of the floating cage is provided elsewhere (6).*

(B) Results from an experiment with green turtle hatchlings in which waves approached from approximately north The range of wave directions during the experiment is indicated by marks outside the edge of the circle. The mean angle of orientation for each hatchling is indicated by a line originating at the circle center (7). Line length is proportional to mean vector length, with a line reaching the edge of the circle corresponding to r = 1 Long lines thus indicate consistent orientation toward a single direction throughout the test period, shorter lines indicate more directional variability F and r values were calculated with the Hotelling test (7). The mean angle of the group (a) is indicated by the large arrow outside of the circle The coastline was approximately parallel to a line defined by the 343°–163° axis (westward, or to the left) but was not visible because we were 28–30 km from shore.

(C) A second experiment with green turtles in which waves approached from approximately southeast. Conventions as in (B).

(D) Summary of the five green turtle and five leatherback orientation experiments conducted 2.0–30.0 km from shore Orientation of each hatchling is plotted as a function of direction of wave approach during the test Direction of wave approach was defined as the mean direction of propagation for all wave types present One or more of three wave types (swells, waves, and wind ripples) were present in all trials. Oceanic swells are generated by prevailing winds over large expanses of water in the open ocean; near Fort Pierce, swells consistently approach from 70°–140° (E–SE) during the summer and are largely unaffected by weather conditions near land Waves generated by local wind patterns were often present and usually moved in directions similar (within 80°) to those of swells A few minutes after abrupt changes in wind direction, wind ripples 1–5 cm in height tracked the new wind direction, while larger "old" waves continued to track the previous wind direction No trials were conducted under stormy conditions when the various wave types could not be clearly resolved or when waves (or swells) exceeded 1.5 m in height The direction of each wave type was measured easily by sighting down the axis of wave propagation with a digital hand-bearing Autohelm™ compass. Because we had no a priori *reason to consider one wave type more important than another, we calculated a mean direction of wave approach for this study. However, hatchlings may actually orient only to the largest waves present (regardless of type) while ignoring the others For a detailed discussion of wave types, see (10).*

Circle-circle correlation analysis (7) indicated that direction of wave approach and hatchling orientation were significantly related (see text). There was no evidence that responses varied with distance from shore.

shown in Figures 1B and C. These results are representative of responses shown by both species. The orientation angles of all hatchlings are plotted as a function of wave approach direction in Figure 1D. Jupp-Mardia circle-circle correlation analysis (7) indicated that wave direction and hatchling orientation were significantly ($P < 0.001$ for each species) related.

None of the hatchlings used in these experiments crawled across the beach; all were taken directly from the nest to the testing site. Thus, turtles can clearly orient in the ocean without crawl experience. But hatchlings might still acquire directional information during the crawl that could alter their orientation while swimming. Turtles with crawl experience, for example, might use different orientation cues than those released offshore without a crawl, or the two groups might use the same guideposts in different ways.

To examine these possibilities, hatchlings with and without beach crawl experience were tested offshore in the floating cage on the night of their expected emergence. Hatchlings were removed from nests before sunset and placed into one of two styrofoam boxes. After sunset both boxes were transported to a nesting beach. The hatchlings in one box were released on dry sand at distances of 10–20 m from the edge of the water. They were permitted to crawl to the wet sand at the edge of the wave wash zone, then retrieved and placed into styrofoam coolers. The lid of the second box was removed during the time the first group was crawling, so that the turtles inside could crawl in the box with a view of the sky. Thus, turtles in both groups crawled, but only one group crawled across the beach.

The orientation of hatchlings with and without a beach crawl was statistically indistinguishable for both green turtles (Fig. 2A–B; Watson test, $U^2 = 0.097$, $P > 0.20$) and for leatherbacks (Fig. 2C–D; $U^2 = 0.045$, $P > 0.20$). Nearly all of the turtles swam in the general direction of approaching waves, suggesting that hatchlings released

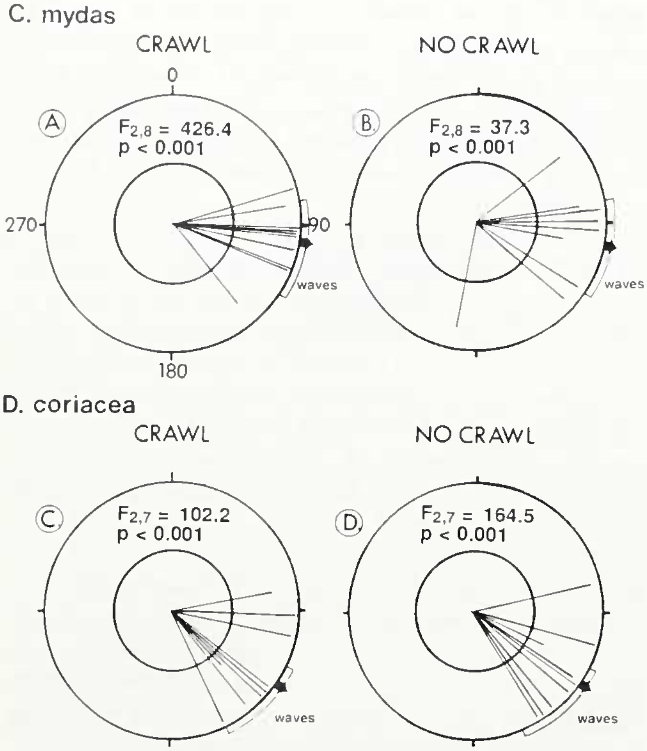

Figure 2. *Results of offshore orientation experiments with hatchlings that had and had not crawled across a beach prior to testing.*

(A) Results of green turtle hatchlings that crawled across the beach. Inner circle indicates r value of 0.5 (outer circle still corresponds to r = 1.0). All other conventions as in Figure 1B. Mean angle for the group is 99°.

(B) Results of green turtle hatchlings deprived of a beach crawl. Conventions as in Figure 2A. Mean angle for the group is 100°.

(C) Results of leatherback hatchlings that crawled across the beach. Conventions as in Figure 2A. Mean angle for the group = 124°.

(D) Results of leatherback hatchlings deprived of a beach crawl. Conventions as in Figure 2A. Mean angle for the group = 124°.

offshore orient toward waves regardless of whether they first experienced a beach crawl.

To study the relationship between turtle orientation and waves more rigorously, we monitored the orientation of hatchlings swimming in a wave tank. Under dim light, each turtle was tethered to a central post (made of nylon fishing line strung vertically from the bottom of the tank to a rod across the top). Thus, hatchlings could swim in any direction in the wave tank but could not contact the sides.

All lights were then turned off except for a single infrared source [a Kodak darkroom light with a 40 W bulb covered by an Edmunds infrared transmitting filter (#8247-29-1)]. After a 5-min acclimation period, an observer using a night vision scope recorded the orientation of each hatchling at 30-s intervals for 5 min; these measurements were used to calculate the mean angle and vector for each turtle (7). One group of hatchlings (for each species) was tested with the wave tank motor off so that no waves were generated and the room was silent. A second group was tested with the motor running but the drive disconnected so that hatchlings were exposed to motor sounds and vibrations, but not to waves. The third group was tested with the motor on and the drive engaged so that waves were generated.

Results for green turtles and leatherbacks were qualitatively identical. Neither species was significantly oriented in the absence of waves (Fig. 3). When waves were present, however, the hatchlings oriented toward the direction of wave approach (Fig. 3). These experiments confirmed that hatchling sea turtles can use waves as an orientation cue, even in the absence of visible light.

Our results suggest that sea turtle hatchlings sequentially employ two separate orientation systems, each based on different cues. While on the beach, hatchlings find the sea by seeking out bright, open horizons (1, 2). Our field and wave tank experiments provide evidence that hatchlings released in the ocean orient by swimming toward waves. Because sea-finding orientation is not a prerequisite for wave orientation, the land and sea orientation systems can function independently.

Our results do not demonstrate, however, that the two systems never interact under natural conditions. Immediately after entering the sea, for example, hatchlings might use visual cues to establish an offshore course. Later, when visual contact with land is lost, hatchlings could maintain their orientation by swimming at a fixed angle relative to waves. Visual cues experienced during either the beach crawl or near land might also be critical for hatchlings that emerge on nesting beaches surrounded by exceedingly calm seas. Additional experiments are required to determine whether the land and sea orientation systems are completely independent under all conditions, or if they interact in some way as hatchlings migrate away from land.

During daylight hours, especially near shore, local winds can generate waves which induce hatchlings to swim in directions other than directly offshore (Fig. 1D). However, Florida sea turtle hatchlings nearly always emerge from nests and enter the sea shortly after dark (8, 9). Oceanic swells, produced by prevailing easterly winds, are the prevalent waves during this time (10). The swells move toward the Florida coast, where the propagation direction becomes oriented perpendicular to the shore as waves enter shallow water and approach a beach (11). Wave propagation direction thus provides a consistent, reliable cue for offshore orientation during the time hatchlings usually enter the ocean.

Several marine molluscs (12, 13) and crustaceans (14, 15) can orient using waves or wave surge in shallow water near shore. Sea turtle hatchlings, however, continued to

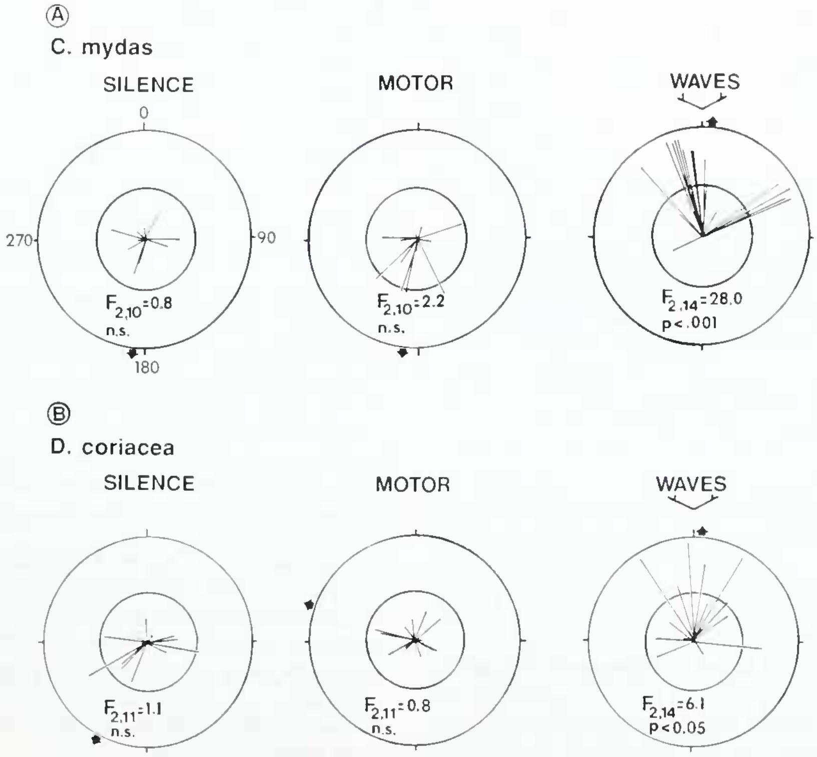

__Figure 3.__ Results of the wave tank experiments. (A) Results of green turtle hatchlings. (B) Results of leatherback hatchlings. All conventions follow those in Figure 2A. For each species, "Silence" indicates results of hatchlings tested with the wave tank motor off. "Motor" indicates the motor was on but the drive was disconnected so no waves were produced. "Waves" indicates results of hatchlings tested when waves were generated; waves approached from 0° (top of the diagram). Each species was significantly oriented only when waves were present. The mean angles of the two wave groups (far right, upper and lower) were directed almost straight toward the direction of wave approach.

WAVE TANK: The wave tank, located at the Florida Institute of Technology in Melbourne, Florida, was 9.1 m in length, 0.9 m high, and 0.6 m in width. It was filled with water to a depth of 0.5 m. A paddle at one end was driven by a DC motor. A sloping plywood platform 4.9 m in length at the opposite end absorbed wave energy and minimized wave reflection. Hatchlings were tethered (see text) so they could swim in any direction while an observer watched with a night vision scope through a circular opening in a styrofoam sheet wedged across the top of the tank. The styrofoam was marked in 5° increments, providing a reference for determining orientation. Throughout the experiments we alternated between trials under the three treatments described in the text (silence, motor, waves) in semi-random order (blocks of 1–3 trials under one condition were followed by similar blocks of trials under the other two conditions). Each hatchling was tested only once under one of the three conditions.

Waves generated in green turtle experiments were 5 cm (peak to trough) in height (frequency about 40 waves/min.). Waves in leatherback experiments were 2–3 cm in height, also at 40 waves/min. We have observed waves of these approximate heights and frequencies at sea on calm days during the summer at Fort Pierce. Natural waves during the summer are only occasionally smaller than this and are often considerably (2–50 times) larger.

swim toward waves even when out of sight of land (beyond about 18 km from shore), indicating that wave propagation direction can be used by animals as a cue for orientation in the open ocean. This cue might well be used by other long-distance ocean migrants such as fish and cetaceans.

The responses of the hatchlings reported here must be viewed as the earliest manifestations of a sophisticated orientation system, one that allows these animals as adults to complete migrations between nesting sites and feeding grounds located hundreds or thousands of kilometers apart (16, 17). Further ontogenetic analysis may provide insight into how an orientation system that initially guides hatchlings offshore develops into one that provides adults with the ability to complete complex navigational tasks.

Acknowledgments

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Abstracts of Papers Presented at the General Scientific Meetings of The Marine Biological Laboratory

August 20–22, 1990

Abstracts are arranged alphabetically by first author within the following categories: cell motility, developmental biology, ecology and population biology, fertilization and early development, mariculture and the marine environment, neurobiology and biophysics, physiology and behavior, and sensory biology. Author and subject references will be found in the regular volume index in the December issue.

Cell Motility

Detailed observations on the migration of Limulus *amoebocytes by video microscopy.* E. L. BEARER, M. STROUT, AND DAVID DEMERS (Dept. of Biochem., University of California, San Francisco).

The amoebocyte—the blood cell of the horseshoe crab—is a composite neutrophyl-thrombocyte capable of either directed migration and phagocytosis or activation, degranulation, and clot formation. The migratory behavior has been difficult to study because the amoebocyte is extremely sensitive to endotoxin, rapidly degranulating in its presence. By putting fresh cells upon a lawn of spread amoebocytes in serum on baked glass coverslips, one of us (E.L.B.) has observed in detail the migratory behavior of these cells. The amoebocyte crawls at a maximum rate of 20–30 μm/min. It extends long filopodia rich in actin filaments in the general direction of movement. Initially, these extend above the surface of the substrate, then come to rest upon it. An attenuated sheet of membrane advances between forward-most filopods, and is subsequently inflated by cytoplasm. By phalloidin staining, this attenuated membrane does not contain actin ruffles, and no retrograde movement can be observed. An apparent contractile event squeezes the nucleus and posterior cytoplasm containing the large granules forwards. During this process, filopods that have extended, but no submerged in membrane, are collected to the rear, such that the cell resembles a pin cushion. By phalloidin staining, actin filament bundles extend from the leading filopods along the ventral surface of the cell to the posterior pin cushion. Filopods initially extend at the rate of 3–5 μm/s, but sufficient G-actin for such a rate of polymerization could not be detected. Amoebocytes will continue to crawl for 15–30 min after being permeabilized briefly with 0.1 mg/ml saponin in a magnesium-EGTA buffer. If 2 m*M* ATP is added, some cells can continue to crawl for up to 5 h. These results suggest that the filopods are extended by an ATP-dependent sliding process rather than by polymerization of new filaments, supporting a role for actin-based motors in the leading edge. To identify such motors, we have adopted a biochemical approach and have isolated a fraction enriched in actin-dependent ATPase activity that retains actin filaments in an *in vitro* motility assay. By Coomasie staining of protein gels, this fraction contains no proteins in the molecular weight range of myosin II (220 kDa), but has several bands.

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Effects of cAMP-dependent protein kinase inhibitor on organelle movement in Y-1 adrenocortical tumor cells. GEORGE M. LANGFORD (Department of Physiology, University of North Carolina, Chapel Hill, NC 27599), EDWARD E. LEONARD, DIETER G. WEISS, AND SANDRA A. MURRAY.

The saltatory motion of lysosomes in Y-1 adrenal cells was quantitatively analyzed in control populations and in cells into which protein kinase inhibitor (PKI) was introduced by electroporation. Organelle motion was studied to determine whether a change in movement pattern occurred in the presence of a factor that suppressed steroidogenic activity. Organelle motion was analyzed from the positional data obtained at 100 ms time resolution. The movement of lysosomes (0.5–0.8 μm diameter) was the Interrupted Motion Type II as defined by Weiss *et al.* (1986, *Cell Motil. Cytoskel.* **6**: 128–135). In control populations of cells, these organelles exhibited periods of rapid directed movement interrupted by pauses that were most often followed by a reversal of direction of movement along the same or adjacent parallel tracks. The average velocity of lysosome movement in both the anterograde (toward the cell periphery) and the retrograde (toward the nucleus) directions was 1.2 ± 0.8 μm/s with a V_{max} of 2.1 ± 0.7 μm/s. The average duration of pauses was 17 ± 5.3 s, while the average duration of movement was 8 ± 4.9 s. In PKI-treated cells, the average and maximum velocities (1.11 ± 0.5 and 2.73 ± 0.8 μm/s, respectively) were not significantly different from those measured in control cells. However, the ratio of the average duration of pauses (4.7 ± 3.3 s) and the average duration of movement (3.5 ± 2.7 s) in the PKI-treated cells was significantly different. We conclude that PKI had no apparent effect on the motors of organelle movement because the maximum and average velocities were not affected. The reduction in the duration of pauses may be due to changes in the degree to which the cytomatrix is crosslinked. We interpret these results to mean that phosphorylation increases the degree of cytomatrix crosslinkage and that such linkages influence the pattern of organelle motion.

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Reorganization of cytoskeleton during cell fusion induced by electric field. Q. Zheng and D. C. Chang (Dept. of Mol. Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030).

Cultured mammalian cells can be fused with high efficiency by applying a pulse of radio-frequency electric field. We have used the electrofused CV-1 cells to study the reorganization of cytoskeletal proteins during the various stages of cell fusion. The structures of microtubules (MTs) and the intermediate filament vimentin were studied by means of immunofluorescence microscopy. The structure of F-actin was examined using rhodamine-labeled phalloidin. We observed that stress fibers disappeared quickly after the initiation of fusion. Later, F-actin bundles were formed at the cell peripheries or at the edges of cytoplasmic bridges; these bundles may help to extend the cytoplasmic bridges. The stress fibers reappeared about 2 h after the electrical treatment. MTs began to extend into the cytoplasmic bridges within a few minutes after the initiation of cell fusion. Later, a network of parallel MT bundles appeared between the adjacent nuclei of the fusing cells. After the nuclei of the fusing cells had aggregated, the MT networks reorganized into a network similar to that of the control cells with a single MT organizing center. A high concentration of colchicine (0.1–1 mM) could disrupt MT bundles and block nuclear aggregation in some of the fusing cells. Colchicine treatment could also impede the merging of cytoplasms. The vimentin proteins extended into the cytoplasmic bridges during cell fusion, but with a slight time delay. The distribution of vimentin proteins generally followed that of MTs. Thus, the formation of parallel MT bundles in fusing cells may provide the mechanical links for nuclear aggregation.

Elasmobranch eye lens: actin and UV radiation. Seymour Zigman (University of Rochester School of Medicine), Nancy S. Rafferty, and Kris C. Lowe.

The contribution of actin integrity to the stability of dogfish (*Mustelus canis*) and skate (*Raja erenacia*) eye lens epithelial cells was investigated. The role of near-UV radiation as a cytoskeletal actin-damaging agent was further investigated. Three procedures were used to analyze fresh elasmobranch eye lenses that had been incubated for up to 22 h *in vitro*, with elasmobranch Ringer's medium, and with and without exposure to a near-UV lamp (emission at 365 nm ± 30 nm; irradiance of 4 mW/cm^2). The lenses were observed histologically with phalloidin-rhodamine specific staining; by transmission electron microscopy, with and without gold-labeled antibodies; and by polyacrylamide gel electrophoresis with immunoblotting. In addition, solutions of purified polymerized rabbit muscle actin (supplied by Dr. Thomas Pollard, MBL) were exposed to the same UV conditions, and depolymerization was assayed by ultracentrifugation and high pressure liquid chromatography. Actin monomer increased due to UV exposure from 3 to 18 h.

We found that skate and dogfish lenses developed superficial opacities due to UV exposure. In whole mounts of lens epithelium, actin filaments in the basal region of the cells were broken down within 18 h of UV exposure. TEM confirmed the breakdown of actin filaments due to UV exposure. PAGE and immunoblotting positively identified actin in these cells. Direct exposure of purified polymerized actin in polymerizing buffer, led to a 10 to 20% increase in actin monomer in the test solutions within 3 to 18 h, whether assayed by ultracentrifugation or HPLC.

We conclude that elasmobranch lens epithelial cells contain UV labile actin filaments, and that near-UV radiation, as is present in the sunlit environment, can break the filaments down in these cells. Furthermore, breakdown of purified polymerized actin does occur due to near-UV light exposure. While the damage appears to be a direct effect of the UV radiation in the actin, the damage could be at the sites at which actin bind to the supporting cell architecture.

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Developmental Biology

S.E.M. observations of early cleavage in Hoploplana inquilina. John M. Arnold (University of Hawaii), Hyla C. Sweet, and Barbara C. Boyer.

Many "lower" invertebrates display "mosaic development" in which the fates of the early blastomeres are determined very early in ontogeny. By studying very primitive animals, which can be thought of as being "frozen in evolution," we can gain insight into the origin of mosaic development.

The current observations were made over the last seven summers on the primitive flatworm *Hoploplana inquilina* using standard fixation techniques for scanning electron microscopy. Our results indicate that some embryos, when viewed from the side or vegetral pole, show furrowing beginning unipolarly at the animal pole and proceeding to the vegetal pole. This results in the two blastomeres being temporarily connected by a large cytoplasmic bridge that is eventually reduced to a mid-body. This mid-body is later lost, but the cells remain with close membrane contact. The bridge is covered with cytoplasmic protusions and folds, which appear to indicate subsurface tensions. It has been demonstrated in echinoderm embryos that the position of the cleavage furrow is determined by the position of the mitotic apparatus. Further, in asymmetrical cleavage, the yolk concentration in the vegetal region of the egg may displace the influences of the M.A. so that the microfilaments causing cytokinesis form at the animal pole and progress vegetally around the zygote to eventually meet at the vegetal pole.

Cleavage and mosaic development are highly variable in these embryos; both bipolar and unipolar cleavage occur in the same batch of eggs. This may indicate that early development in this flatworm is a constant, ongoing evolutionary experiment.

Supported by a NSF grant DCB 8817760 to Barbara Boyer.

Morphogenesis of ascidian ampullae and polarized movements of tunic extracellular matrix components along ampullae. William R. Bates (Dept. of Biology, Carleton University, Ottawa, Canada).

The ampullae of solitary ascidians are epidermal structures that attach the juvenile to specific kinds of substrates and are thought to secrete the extracellular matrix (ECM) components of the tunic. Each ampulla grows out from the body epidermis as a fluid-filled tube, surrounded by a single layer of cells, and encased within the tunic ECM. In the present study, the movements of contractile rings along ampullae 200 to 800 μm in length were investigated using *Molgula manhattensis* (a urodele species) and *M. provisionalis* (an anural species). Similar results were obtained for both species. Within the ampullar lumen, near the base of each ampulla and in close proximity to the developing zooid (at days 2, 3, and 4), an oscillating bubble-like region formed before each contraction wave. Each of these regions functioned autonomously for several days before their pulsations stopped. These regions may be localized centers of varying hydrostatic pressure that may trigger the polarized movements of the contractile rings in a proximal to distal direction. Contractile rings were produced at regular time intervals over several days and could be blocked with cytochalasin D (at 10 μg/ml). Previous TEM studies of an anural species, *Molgula pacifica,* demonstrated a circumferential pattern of microfilaments in the basal cytoplasm of ampulla cells (Bates and Mallett, *Can. J. Zool.* in press). Together these results suggest that ampullar contraction waves are mediated by microfilaments. When clusters of chalk particles were positioned on the tunic ECM near the tips of day 2 ampullae, the particles were translocated in a distal to proximal direction and, by day 3, decorated the surface of the tunic that encased the zooid. These experiments suggest that tunic ECM components are synthesized by ampullar epidermal cells, and that there is a polarized movement of these components along ampullar surfaces.

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Macromere control of early development in the polyclad flatworm Hoploplana. BARBARA C. BOYER (Union College, Schenectady, NY 12308).

Either one, two, three, or four macromeres were deleted from eight-cell stage embryos of the polyclad flatworm *Hoploplana inquilina,* with multiple deletions involving various combinations of cross-furrow (1B and 1D) and non-cross-furrow (1A and 1C) cells. Normal Müller's larvae developed in 28% of embryos from which one blastomere was removed, regardless of whether it was a cross-furrow or a non-cross-furrow macromere. Deletion of more than one macromere never produced normal larvae.

Although first quartet micromere deletions produced loss of eyes (Boyer, 1989, *Biol. Bull.* **177**: 338–343), macromere ablations usually resulted in supernumerary eyes. As more macromeres were removed, the number of larvae with extra eyes also increased, with cross-furrow macromere deletions producing additional eyes more often than non-cross-furrow deletions (*e.g.,* 52% with loss of 1B and 1D *versus* 22% with deletion of 1A and 1C).

Similarly to first quartet deletions, as increasing numbers of macromeres were removed, the frequency of bilaterally symmetrical larvae with Müller's morphology declined. Most of the abnormal larvae were "swollen," consisting of lobeless spheres with undifferentiated masses of internal cells surrounded by a cavity and usually possessing supernumerary eyes. However, bilaterally symmetrical larvae developed more frequently when non-cross-furrow blastomeres were deleted. For example, deletion of one cross-furrow cell produced approximately the same percentage of Müller's-type larvae (64%) as deletion of both non-cross-furrow macromeres (62%). Similarly, deletion of 1B and 1D, and deletion of three macromeres including 1A and 1C, led to 19% and 18% bilaterally symmetrical larvae, respectively. Loss of all four macromeres produced 100% radial or asymmetric larvae. These results suggest that determination of symmetry in *Hoploplana* involves a mechanism similar to that of the equally cleaving mollusks in which micromere-macromere interactions specifically involving a cross-furrow macromere establish bilateral symmetry and determine the dorsal quadrant.

This work was supported by NSF grant DCB-8817760.

Effects of tumor promoters (okadaic acid, TPA) and anticarcinogens (nicotinamide, sarcophytol A) on Arbacia *development.* KRYSTYNA FRENKEL[1], HIROTA FUJIKI[2], ALBERT GROSSMAN[1], AND WALTER TROLL[1] ([1]New York University Medical Center and [2]National Cancer Center Research Institute, Japan).

The sequence of cell divisions that follows fertilization of sea urchin eggs by sperm may serve as a model for tumor promotion and progression in mammalian systems. One observation that supports such a notion is that H_2O_2 is produced immediately after fertilization and also is one of the earliest effects elicited by tumor promoters in human neutrophils. Two types of tumor promoters were used: okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, and TPA (12-O-tetradecanoylphorbol-13-acetate), an activator of protein kinase C. Okadaic acid (in the range of 10^{-8} *M*) and TPA (in the range of 10^{-9} *M*) inhibited development of sea urchin embryos when given 15 min prior to fertilization. Although fertilization and the subsequent 3 to 4 divisions appeared to be normal, development was significantly retarded thereafter. Lower concentrations of these tumor promoters seemed to accelerate development, whereas higher concentrations were more toxic.

The anticarcinogens nicotinamide and sarcophytol A inhibited oxygen radical release by tumor promoter-activated human neutrophils in a time- and dose-dependent manner. Preliminary evidence suggests that such anticarcinogenic agents may also be effective in overcoming the inhibitory action of tumor promoters on sea urchin development. That ability may become the basis for screening the agents for their potential anticarcinogenic activity.

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Morphology and reproductive tract development in winter and summer populations of the male spider crab Libinia emarginata: *a proposed life history and regulatory mechanism.* HANS LAUFER, ELLEN HOMOLA, ARMAND M. KURIS, AND AMIR SAGI (Molecular and Cell Biology, University of Connecticut, Storrs, CT).

We investigated a possible role for the mandibular organ (MO) and its secretion, methyl farnesoate (MF), in the regulation of reproduction and morphogenesis. Two different subpopulations were observed in winter (1989) and summer (1990) populations, with respect to relative claw size (propodus/carapace). The shell texture of small-clawed males was always pubescent, whereas large-clawed males were either pubescent, with velvety carapaces indicating a recent molt, or abraded, with a worn carapace, indicating a prolonged intermolt. All males had sperm and were in their intermolt phase. Abraded winter males were the largest, with heavily calcified carapaces. In the summer, no differences in calcification were noted, and abraded crabs of different sizes were present. Variation in carapace, from smooth to velvety pubescent, was noted. The reproductive system was significantly more developed in abraded males, and reproductive system size correlated well with body size. Large-clawed pubescent crabs had small reproductive systems that were not correlated to their body size. Small-clawed males possessed a small reproductive system that was correlated with their body size. MF in the hemolymph and its synthesis by the MO was two to five times higher in animals with the larger reproductive systems.

Mature *L. emarginata* males molt in the fall. We suggested that the abraded crabs were in their terminal molt, and that they transformed in the fall of 1988 (or before). Full reproductive tract development followed. The pubescent large-clawed males, having similar body sizes to the abraded males, seem to have had their terminal molt in the fall of 1989 and still have a growing reproductive system. Small-clawed males continue to molt. The evidence strongly supports a role for MF as a gonadotropin, which directs the growth of the reproductive system; it may also play a role in the development of male polymorphism.

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Metalloproteinases of the developing sea urchin embryo. J. QUIGLEY (Pathology, SUNY, Stony Brook, NY 11794), R. S. BRAITHWAITE, AND P. ARMSTRONG.

Proteolytic enzymes play an active role in inflammation and in tumor cell metastasis. Of particular importance are the enzymes that degrade the basal lamina, because it is a major barrier to cell motility. Gelatinases play a key role in basal lamina degradation, because they degrade collagen type IV, a major constituent of the basal lamina. We have investigated the activity of gelatinases in *Arbacia* embryos to observe their role in the extracellular matrix remodelling that occurs in normal embryonic development. Gelatinase activity is detected by SDS-polyacrylamide gel electrophoresis, with gelatin incorporated into the resolving gel. Washing with Triton removes the SDS, and an incubation period immediately thereafter allows the enzymes to degrade the gelatin. This activity is revealed subsequently by bands of clearance in Coomassie blue stain. We find four prominent zones of lysis (gelatinases) at approximate molecular weights of 50, 45, 37, and 33 kDa. As the embryos advance through the blastula, gastrula, and prism stages, the 37 and 33 kDa bands increase in activity, and the 44 kDa band decreases in activity. The activity of these enzymes is increased after an incubation with 1 mm APMA, an organomercurial that activates mamallian gelatinases. These enzymes are completely inhibited by 1 mm 1,10-phenanthroline and EDTA, and are unaffected by the serine protease inhibitor PMSF, demonstrating that they are metalloproteinases. The involvement of these enzymes in embryonic extracellular matrix remodelling is under study.

The repetitive calcium waves in the fertilized ascidian egg are initiated in the vegetal hemisphere by a cortical pacemaker. J. E. SPEKSNIJDER (Dept. Exp. Zoology, University of Utrecht, The Netherlands).

Ascidian eggs respond to fertilization with a series of calcium pulses that continues until meiosis is completed (Speksnijder *et al.*, 1989, *Dev. Biol.* **135**: 182–190). In the egg of *Phallusia mammillata,* the first large (activating) pulse is initiated by the sperm at its entry point, usually in the animal hemisphere, and spreads across the egg as a wave (Speksnijder *et al.*, 1990, *J. Cell Biol.* **110**: 1589–1598). The remaining calcium pulses also travel as waves, but their initiation point is located mainly near the vegetal pole, where the developmentally important, mitochondria-rich myoplasm is concentrated (Speksnijder *et al.*, 1990 *Dev. Biol.* **142**, in press). The object of this study is to determine whether the pacemaker of these calcium waves is associated with the myoplasm, or located in the vegetal cortex underlying the myoplasm. Therefore unfertilized eggs were centrifuged at $2000 \times g$ for 5 min to displace their myoplasm. In DIC optics, three cytoplasmic layers are visible in such centrifuged eggs: a centrifugal yolk zone, a myoplasmic zone rich in mitochondria, and a clear zone. Vital staining of mitochondria with di0C2 (0.5 μg/ml, 10 min), and of DNA with H33342 (10 μg/ml, 10 min), reveals that the myoplasm is displaced randomly with respect to the animal pole, which is marked by the female chromosomes. The three cytoplasmic zones remain clearly distinguishable for at least 1 h after centrifugation, and no visible rearrangements of the zones take place at fertilization. Imaging of the cytosolic calcium in 14 stratified, aequorin-injected eggs reveals a series of calcium waves following fertilization. About 60–70% of these waves start near the vegetal pole, which corresponds to the number of waves previously found to start near the vegetal pole in non-centrifuged eggs. In contrast, only 20–30% of the waves start in the vicinity of the displaced myoplasm. I conclude that the main wave initiation site is not displaced by the centrifugal forces used, which suggests that a cortical component located in the vegetal hemisphere is involved in initiating these repetitive calcium waves in the fertilized ascidian egg.

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Single and multiple micromere deletions in first quartet embryos of Ilyanassa obsoleta. HYLA C. SWEET AND BARBARA C. BOYER (Union College, Schenectady, NY 12308).

Following the deletion of the 1a or 1c micromeres of eight-cell *Ilyanassa* embryos, Clement (1967, *J. Exp. Zool.* **166**: 77–88) found that, at seven days, the veliger larvae were missing the left or right eye, respectively. To determine whether the experimental larvae can replace the missing eye, as is the case in *Bithynia* (van Dam and Verdonk, 1982 *Roux's Arch. Dev. Biol.* **191**: 112–118), *Ilyanassa* embryos were raised to an average of 14 days after the deletion of 1a or 1c. None of the veligers formed a second eye, indicating that eye development in *Ilyanassa* is not as regulative as it is in *Bithynia*.

Combinations of first quartet micromeres were deleted to further test the mosaic *versus* regulative nature of the *Ilyanassa* embryo. The removal of the whole first quartet resulted in several larvae that had stump-like structures instead of a velum, suggesting that the first quartet is determined at the eight-cell stage to form head structures, with little contribution from other blastomeres.

Clement found considerable variation in 1b-deleted veligers, but little difference between 1d-deleted and control larvae. The results of experiments in this study, in which both 1b and 1d were removed, support Clement's suggestion of a general unstabilizing effect created in 1b-deleted larvae. However, the degree of variation was higher than in Clement's single 1b deletions and occurred mainly in the size and shape of the velum and in the number of eyes. This suggests that the deletion of 1b and 1d together causes a loss of regulation among the first quartet micromeres. Because little variation was found among multiple micromere deletions involving 1d, and either 1a or 1c, a destabilizing effect may be created by the loss of 1d only when it is deleted with 1b. Thus 1b and 1d may work together during the early development of *Ilyanassa* to establish some sort of stability, or regulation, in forming the head structures.

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Ecology and Population Biology

Feeding and predation rates of winter flounder, Pseudopleuronectes americanus, *and blue crabs,* Callinectes sapidus, *in an eelgrass bed in Waquoit Bay.* ROBERT BILLARD (Marine Biological Laboratory), LAURA LYNCH, LINDA A. DEEGAN, JOHN T. FINN, AND SUZANNE G. AYVAZIAN.

Development around Waquoit Bay, Massachusetts, is adding nutrients that may adversely effect young of the year (YOY) finfish and shellfish populations. This nutrient deposition causes dense macroalgal growth, which may inhibit these animals from finding adequate protection from predation and food supply. A field study was conducted to investigate the impact of macroalgae in an eelgrass habitat on the feeding habits of, and the predation rates on, YOY winter flounder and blue crabs.

Four (25×25 m) plots of varying macroalgal density were established. First, algae were removed from one plot (removal) and added to a second plot (addition). A control plot with normal amounts of algae and a disturbance control plot from which the algae were removed and then returned, were also sampled. Predation rates were determined by tethering YOY of each species in the four plots for 6 (flounder) or 24 h (blue crabs). At the end of the period, the presence or absence of these animals was noted. Absence was assumed to be due to predation. Feeding habits of the two species were determined by placing starved animals in the plots and allowing them to feed for 2 h.

We conclude that macroalgal density may influence the predation rates on, and the feeding habits of, YOY winter flounder and blue crabs. The predation rate on winter flounder was significantly higher in plots with high algae concentrations. There was no observable difference in predation rates on the blue crab. Initial results indicate that winter flounder fed when dissolved oxygen and temperature were within tolerance limits. The blue crab feeding experiment was done in late summer, and very few crabs fed. We believe feeding stopped in the late summer due to anoxic conditions and high temperatures.

Stochastic and deterministic models of niche displacement. John F. Boyer (Union College, Schenectady, NY 12308).

I used computer simulation to study the interaction of two species, each of which had two phenotypes determined by one diallelic locus. One phenotype was a generalist predator on five different prey types; the other was a specialist on only one kind of the prey. The fitness (reproductive rate) of each phenotype depended on its predation success. In the deterministic experiments, a second-order difference equation was used to calculate predation rates, and genotypic frequencies at birth were always in Hardy-Weinberg proportions. In the stochastic experiments, predation was a consequence of random encounters, and genotypic frequencies were subject to genetic drift. When the two species were allopatric (non-competing), the specialist individuals persisted at a (usually) low equilibrium frequency in the deterministic experiments, and were only rarely persistent in the stochastic experiments, in which fixation of the generalist allele was the predominant outcome. When the two species were sympatric and the generalists of both species competed for one or more prey types, the specialist individuals attained a higher average frequency than did their counterparts in the non-competing populations, thus showing niche displacement. The predation level (or efficiency), the magnitude of the satiation (or handling-time) parameters, and the resource coarseness (few large prey *versus* many small prey) all had a significant effect on the equilibrium frequencies or fixation probabilities. Almost all combinations of values for the five major experimental variables resulted in niche displacement, but the frequency-dependent selection forces were not strong enough to prevent allelic fixation in the majority of the stochastic populations. These results confirm the theoretical ubiquity of niche displacement, but also suggest that the magnitude of this displacement is likely to be sufficiently small that clearly discernable instances of this phenomenon will be quite rare in nature.

This research was supported in part by a grant from Union College.

*The effects of macroalgae on the abundance of eelgrass (*Zostera marina*) in the Waquoit Bay Estuary.* Heidi E. Geyer (The Marine Biological Laboratory), Linda A. Deegan, Jack T. Finn, and Suzanne G. Ayvazian.

Within the last 20 years, estuarine eelgrass (*Zostera marina*) bed communities within the Waquoit Bay area have diminished in size and abundance. Concurrently, macroalgal abundance has increased. The impact of macroalgal development on *Zostera marina* in the Waquoit Bay Estuary was examined.

Four plots (25 × 25 m) of varying macroalgal density were established. Macroalgae were removed by SCUBA from one plot (removal plot) and placed in another (addition plot). A disturbance control plot was established by removing the algae and replacing them in the same plot. A control plot was established in which no algal manipulation was done. Macroalgal density varied significantly in the removal and addition plots. The abundance of eelgrass was determined by counting tufts at the stem base along 0.5 m × 35 m transect lines within each plot both before (May 1990) and after (August 1990) the manipulation.

Removal of macroalgae from a pre-existing eelgrass bed appears to cause increased tuft abundance in *Zostera marina*. From May to August, the average number of tufts increased significantly in the removal plot and decreased significantly in the disturbance control plot. No significant difference in tuft abundance was seen in either the addition plot or in the control plot. The influence of macroalgae in decreasing eelgrass beds could be the result of direct and indirect effects. Macroalgae can directly over-grow eelgrass. It also causes a strongly anoxic benthic layer, which is difficult for eelgrass to sprout in. Additionally, on sunny days we observed large mats of algae, filled with oxygen bubbles, lift off the bottom and float away, taking rooted eelgrass with them. Experimental results and observations lead us to believe that dense macroalgae mats may contribute to the loss of healthy eelgrass beds in Waquoit Bay.

The effects of macroalgae on the abundance and diversity of free-swimming invertebrates in eelgrass beds of Waquoit Bay, MA. Kristin M. O'Brien (Marine Biological Laboratory) Linda A. Deegan, John T. Finn, and Suzanne G. Ayvazian.

Macroalgae in eelgrass beds of Waquoit Bay may decrease the suitable area of nursery habitats for juvenile fishes by affecting food abundance and availability. The effects of macroalgae on the abundance and diversity of free-swimming invertebrates were studied in a large scale field experiment.

Four 25 by 25 meter plots with varying densities of macroalgae were constructed. A removal plot, containing minimal amounts of algae, an addition plot with algae taken from the removal plot, a control, and a disturbance control were all maintained from June through August. Mobile invertebrates were sampled with activity traps. Six traps were deployed in each plot for 24 h during July and August.

An increase in macroalgal density apparently increases free-swimming invertebrate abundance, but invertebrate diversity is decreased. Typical species found were *Gammarus oceanicus, Erichsonella attenuata, Edotea triloba, Calanoid copepoda,* and *Palaemonetes pugio.* During July, there were significantly fewer invertebrates in the removal plot compared to both the control and addition plots, but during August there was no difference in abundance among the plots. The decreased invertebrate abundance was correlated with decreased algal density. When we combined all of the data we found that invertebrate abundance was inversely correlated with invertebrate diversity. Therefore, free-swimming invertebrate populations, which are important food sources for juvenile fishes, are increased in abundance and decreased in diversity in high density macroalgae areas.

Influence of macroalgae in eelgrass beds on finfish abundance and dissolved oxygen in Waquoit Bay. Rebeka J. Rand (Marine Biological Laboratory), Linda A. Deegan, John T. Finn, and Suzanne G. Ayvazian.

Waquoit Bay is an important estuarine area for resident, catadromous, and seasonally migrating finfish, as well as the nursery habitat for some commercial fish species. An increase of macroalgal abundance has decreased dense eelgrass beds. Therefore, we hypothesize that this macroalgal infestation of *Zostera marina* beds has reduced species diversity, total number, and total biomass of fishes.

Four experimental plots (25 × 25 m) were established: removal, addition, disturbance, and control. The macroalgae were removed from one plot (removal) and placed into the addition plot. Fish were collected using 1 × 1 m fish traps with $\frac{1}{16}$ inch mesh netting. Four traps per plot were taken monthly from May to July. Samples were identified, counted, weighed, and measured.

Our results indicate a greater number and biomass of fishes in the removal plot. Cumulative total fish biomass was highest in the removal plot (n = 33.6 g), followed by the control (n = 25.2 g), disturbance (n = 21.1 g), and addition (n = 17.6 g) plots. The total number of fish was highest in the removal plot (n = 70), compared to the control (n = 24), addition (n = 30), or disturbance (n = 36) plots. There was no trend in the species number among the plots. Juvenile winter flounder were only observed in the removal plot. Fourspine sticklebacks were the dominant species in the samples and were primarily found in the removal plot. Other species showed a mixed distribution among the plots. The observed distribution in fishes may be due to an increase in bare substrate for benthic fishes (*i.e.*, juvenile winter flounder and benthic egglayers) and an increase in habitat for eelgrass dependent species (*i.e.*, fourspine stickleback).

Fertilization and Early Development

Where does the calcium lost by fertilizing Arbacia *eggs go?* I. GILLOT, J. CHRYSTAL, L. F. JAFFE, AND W. M. KÜHTREIBER (Marine Biological Laboratory, Woods Hole, MA 02543).

After eggs of the sea urchin *Arbacia* are fertilized, a calcium wave traverses the eggs at about 14 μm/s. This wave is immediately followed by an exocytotic wave that releases the contents of the cortical granules, resulting in the lifting off of the fertilization membrane. In this study, we have used our recently developed vibrating ion selective probe system (Kühtreiber and Jaffe, 1990, *J. Cell Biol.* **110**: 1565–1573) to investigate which mechanisms are responsible for returning the calcium concentration to its resting level.

Arbacia punctulata eggs were fertilized in artificial seawater (ASW) containing 1 m*M* Ca^{++}. We could measure a calcium efflux during the 215 ± 10 s (S.D.; n = 5) before the signal was lost in the noise, with a peak efflux of about 11 pmol/cm^2/s. The total calcium lost during this period was calculated to be about 11.7%, which is in excellent agreement with the results of Azarnia and Chambers (1976, *J. Exp. Zool.* **198**: 65–78). We also performed measurements on eggs of *Phallusia mammilata*. Although the eggs of this ascidian do not contain cortical granules, we nevertheless measured a post-fertilization calcium efflux with a duration and magnitude similar to that of *Arbacia* (265 ± 18 s; S.D. n = 4).

Moreover, post-fertilization measurements with a proton-selective vibrating probe showed that proton release by exocytosis lasts less than 20 s. Presumably, calcium release by exocytosis would also last for this short period.

In summary, fertilized sea urchin and ascidian eggs use a calcium pump that actively transports intracellular free calcium out of the cell. Only a small part, if any, of the calcium lost from *Arbacia* eggs to the medium can be due to cortical granule release.

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The path of calcium in fertilization and other endogenous oscillations: a unifying view. LIONEL F. JAFFE (Marine Biological Laboratory, Woods Hole, MA 02543).

Throughout the vertebrate line, eggs are activated by a calcium wave that crosses them at about 10 μm/s. In tunicates, as well as mammals, these fertilization waves are followed by a long, periodic series of afterwaves (Speksnijder *et al.*, 1990, *Dev. Biol.* **142**). A growing number of the periodic calcium pulses found in various cultured cells also consist of calcium waves. This abstract makes five postulates. (1) Every calcium pulse takes the form of a calcium wave. (2) Every medium speed (*i.e.*, 3–300 μm/s) calcium wave is initiated and then propagated by two distinct modes of calcium-induced calcium release: first, in the lumenal mode, a slow rise of calcium within the lumen of the endoplasmic reticulum (E.R.) reaches a trigger level that initiates fast, localized release into the cytosol; then the well-known cytosolic mode drives a reaction-diffusion wave across the cell. (3) The velocities (v) of such calcium waves are governed by the Luther equation:

$$v = K\sqrt{\frac{D}{t}}$$

where D is the diffusion constant of an autocatalytic substance (here Ca^{++}), t is the time taken to rise e-fold during the autocatalytic rise, and K is about one (Arnold *et al.*, 1987, *J. Chem. Ed.* **64**: 740–744). (4) During the latent periods that precede fertilization, as well as some agonist-induced oscillations, calcium is pumped into a wave initiation or pacemaker region of the E.R. (5) In fertilization, this calcium comes from the medium via the inner acrosomal membrane of the fused sperm.

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The gigantic germinal vesicles of elasmobranchs. C. D. LEIDIGH, N. H. KIM, R. D. GOLDMAN, A. GOLDMAN, M. V. L. BENNETT (Albert Einstein College of Medicine), AND G. D. PAPPAS.

Germinal vesicles (GVs) of the skate, *Raja erinacea*, are readily visible as small clear regions just beneath the surface of immature and mature oocytes. The GVs are exceptionally large, and their diameters range from 280 to 500 μm in oocytes from 0.7 to 20 mm in diameter. Slightly smaller GVs were seen in the larger (40 mm diameter) oocytes of one specimen of the dogfish shark, *Squalus acanthias*. GVs are readily isolated by dissection and are nearly spherical, very transparent, and quite robust. Light microscopy of isolated GVs and light and electron microscopy of GVs fixed *in situ* revealed structural details. The nuclear envelope is somewhat convoluted and consists of two membranes with abundant nuclear pores about 60 nm in diameter. The inner aspect of the envelope is covered by a thick (50 nm) layer, probably of lamin. The nucleoplasm contains some granular material, but no apparent structure such as nucleoli. DNA staining with Hoechst 33342 or RNA staining with thyazol orange was negative, presumably because the chromatin is dispersed. SDS-PAGE of isolated vesicles reveals several bands between 60 and 70 kDa, which are likely to be lamin, as well as a band at 45 kDa ascribable to actin. The lamin may account for the strength of the GV envelope. Isolated GVs were bathed in fluorescent tracers in elasmobranch physiological saline and penetration and washout observed. The nuclear envelope is permeable to fluoresceinated dextrans of both 10 kDa and 70 kDa molecular size and is less permeable to the larger tracer. Consistent with the permeability to large molecules, we could detect no membrane potential or resistance. These GVs should be a valuable preparation for characterization of properties of the nuclear envelope.

Calcium waves spread beneath the furrows of cleaving Oryzias latipes *and* Xenopus laevis *eggs.* ANDREW L. MILLER (Marine Biological Laboratory), RICHARD A. FLUCK, JANE A. MCLAUGHLIN, AND LIONEL F. JAFFE.

To monitor cytosolic [Ca^{2+}] during cytokinesis, recombinant aequorin was microinjected into eggs of *Oryzias* and *Xenopus*. Photon emission was monitored with an ultra-sensitive imaging photon detector, and the mechanical events accompanying cytokinesis recorded using time-lapse video microscopy. Most of our observations were made on the two furrows that form during the second cleavage of the blastodisc in *Oryzias*, where cytokinesis consists of two distinct processes. In the first, a furrow cuts through the blastodisc from the external surface, cleaving the cytoplasm; in the second, the membranes of the daughter cells "zip up," becoming closely apposed. Both processes begin near the center of the blastodisc, at right angles to the first furrow, and end at the edges of the blastodisc. At 19°C, the second furrow forms in 7 min, begins to zip 3 min later, and finishes zipping 14 min later; the entire process takes 24 min. We observed two distinct calcium waves along the furrow during cytokinesis: the first approximately coincided with the formation of the furrow, and the second approximately coincided with the zipping up of the furrow. That is to say, a calcium hot spot appears near the center of the blastodisc when the furrow begins to form and travels to the edge of the blastodisc, arriving there when the furrow does. A second hot spot appears at the center of the blastodisc when zipping up begins, and it also travels to the edge of the blastodisc. Both waves spread at 0.3–1 $\mu m\ s^{-1}$ and are thus in the class of slow calcium waves, which may be mechanically propagated via stretch activated channels. Similar waves were also detected during first cleavage of *Xenopus* eggs. This is the first report of the visualization of cytosolic calcium changes during cleavage.

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Sea urchin embryos: suitability for exogenous expression of gap junction channels. ALONSO P. MORENO (Albert Einstein College of Medicine) AND DAVID C. SPRAY.

Gap junction channels in vertebrates are formed of a group of homologous proteins termed connexins. The tissue-specific expression of various connexins gives rise to gap junction channels with different physiological properties [*e.g.*, voltage and pH dependence, unitary conductance (γ_j) of the channels]. Because certain tissues may express multiple connexins, it has become desirable to study the properties of connexins expressed individually in exogenous expression systems, including *Xenopus* oocytes. This preparation has certain limitations, such as endogenous connexins, that complicate the interpretation of data from mRNA-injected cell pairs. In addition, single channel studies (where channel currents are detected between two voltage clamped cells) are compromised by the large size and low input resistance of the oocyte. We have attempted to use sea urchin embryos for these studies, because they lack gap junctions at early developmental stages (Spiegel and Howard, 1983, *J. Cell Sci.* **62**: 27–48), and have small blastomeres. Using Lucifer Yellow injection to test for dye coupling, we have found that blastomeres at early cleavage stages (<16-cell) are not coupled following cell division. At later stages (16-, 32-cell), dye transfer is limited to sister blastomeres. The input resistance of the blastomeres (≥800 MΩ) is high enough to calculate the γ_j of the endogenous channels, which averaged about 220 pS (≥16-cell stages). To test for the expression of exogenous mRNAs, mRNA encoding rat connexin32 and transcribed *in vitro*, or, poly A + RNA extracted from the ctenophore *Mnemiopsis*, or deionized water were injected into sea urchin oocytes immediately after fertilization. Subsequent cleavages were judged normal in about 50% of the embryos. Embryos (<16-cell stage) injected with ctenophore mRNA or water showed no dye coupling, whereas in the connexin32-injected blastomeres, dye spread rapidly to sister cells and was detectable throughout the embryo within 5 min (n = ⅝). We conclude that connexin32 is expressible in sea urchin embryos, where the lack of endogenous coupling and small size should permit the characterization of gap junction channel properties inaccessible in *Xenopus* oocytes.

Supported in part by a Grass Foundation Fellowship to A.P.M.

An aerial vibrating probe. RICHARD H. SANGER (Marine Biological Laboratory), ERIC KARPLUS, AND LIONEL F. JAFFE.

We are improving a technique for observing the electrical behavior of a biological system developing in air using the AC Kelvin method. An electrical current travelling through a resistive element generates an electric field outside the element. One can construct a capacitor by placing a conductive plate in the electric field. The charge on the capacitor will be proportional to the local voltage on the resistive element (produced by a current flowing through the element) divided by the distance from the element to the plate. With the aerial vibrating probe, a biological system behaves as a resistive element with a current flowing in it, and a vibrating plate is used as a probe to construct a variable capacitor. Subsurface currents in the biological system generate the electric field to be measured. Vibrating the plate changes the distance between the plate and the source of the electric field, hence changing the charge on the effective capacitor. This changing charge is amplified and processed with a lock-in amplifier. The present device uses a common Field Effect Transistor (FET) as a head stage amplifier. The probe that serves as the detecting plate is the factory-supplied lead on the FET, which is approximately 430 μ in diameter. We have been vibrating the entire FET with a piezoelectric element at about 130 Hz. With a 30-μ vibration at 60 μ from the source, we have already been able to resolve signals of several millivolts, similar to those found on corn coleoptiles in response to light or gravity (Grahm and Hertz, 1962, *Physiol. Plant.* **15**: 96–113). We hope to reduce the size of the probe by a factor of 10 and increase the sensitivity of the system by a factor of 100. This will involve improving the electronics and physical geometry of the system.

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*Gap junction channels in marine embryos: comparison of properties in late blastulae of squid (*Loligo paelei*) and skate (*Raja erinacea*).* D. C. SPRAY (Albert Einstein College of Medicine), A. C. CAMPOS DE CARVALHO, A. P. MORENO, E. SCEMES, C. LEIDIGH, N. H. KIM, G. D. PAPPAS, AND M. V. L. BENNETT.

Gap junctions are abundant in most early embryos; their role, presumably, is to facilitate the exchange of developmentally relevant molecules between the differentiating cells. In previous studies, the physiological properties of gap junctions in ascidian, amphibian, and teleost blastulae have been characterized. In all these groups, gap junctions exhibit voltage sensitivity; *i.e.*, a voltage imposed across the junctional membrane closes the channels. The sensitivity in amphibian and ascidians, but not in teleosts, is great enough to suggest that resting potential differences could act to establish developmental compartments. We therefore examined other embryos. We have now recorded currents through gap junction channels from pairs of cells dissociated from late blastulae of skate (*Raja erinacea*) and squid (*Loligo pealei*) using the dual whole cell recording method with patch pipettes. In skate, we recorded from 18 cell pairs; in the 8 pairs that were coupled [*i.e.*, junctional conductance (g_j) was greater than 10 pS] g_j ranged from 2 to 20 nS, with a mean of 10 nS. In squid, 18 of 23 cell pairs were coupled, with a mean g_j of 13.5 nS. In neither cell type was there a marked degree of voltage dependence. In both types, g_j was reversibly reduced by exposure to 2 mM halothane in the bathing solution. At low values of g_j, single channel currents were observable from which the unitary junctional conductance (γ_j) was calculated. For skate, γ_j values peaked near 100 pS; for squid, γ_j was much larger, with most values above 250 pS. The skate γ_j is similar to that recorded from cells expressing connexins of adult mammals, whereas the squid γ_j is substantially larger. The larger γ_j for the squid junctions is consistent with their larger channel diameter inferred from previous permeability measurements.

Cyclic AMP-dependent phosphorylation of dynein heavy chains in Mytilus edulis *sperm flagella.* R. E. Stephens and G. Prior (Marine Biological Laboratory, Woods Hole, MA 02543).

Lateral ciliary activity in *Mytilus edulis* gill is mediated by serotonin through the cAMP-dependent phosphorylation of presumptive dynein light chains. Because *Mytilus* sperm are also reported to be serotonin-activated, we investigated cAMP-dependent phosphorylation of sperm dynein. Sperm were decapitated by homogenization and the flagella were recovered by differential centrifugation. The flagella were permeabilized with 0.012% NP-40 and then incubated with γ-^{32}-P-labeled ATP, either with or without added cAMP. The reaction was stopped, and the membranes were removed by extraction with 0.25% NP-40. Outer arm dynein was produced by extracting the resulting 9 + 2 axonemes with 0.6 *M* NaCl for 15 min on ice, while inner arm dynein was obtained by a second extraction with 0.6 *M* NaCl containing 0.25% NP-40. The dynein fractions were analyzed by sucrose gradient centrifugation, SDS-PAGE, and autoradiography. Each fraction represented >45% of the total ATPase. The dynein sedimented at 18-21S and consisted of equimolar α and β heavy chains, intermediate chains of 95 kDa and 80 kDa, and three light chains. No light chain phosphorylation was observed. However, the α heavy chains of both the outer and the inner dynein arm fractions were singularly and equally labeled. Phosphorylation occurred maximally at cAMP levels above 1 micromolar; cGMP was also effective, but at >10-fold higher levels. The phosphorylation was extremely rapid, highly stable, and calcium-independent. Photocleavage at 360 nm, in the presence of vanadate and Mg-ATP, yielded products with molecular weights of 225 and 215 kDa (HUV fragments) and 185 and 175 kDa (LUV fragments). Phosphorylation occurred exclusively on the 185 kDa LUV fragment, establishing its derivation from the α heavy chain and arguing against multiple phosphorylation sites. Dynein α heavy chain phosphorylation was similarly obtained with *Spisula solidissima* sperm, also known to be serotonin-activated. These are the first definitive examples of dynein heavy chain phosphorylation in response to cAMP.

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Gossypol-binding proteins from marine species. Hiroshi Ueno (Rockefeller University), Sheldon J. Segal, and S. S. Koide.

During the past few years, various biological activities of gossypol have been explored. Besides male antifertility or antispermacidal activity, we have reported antiparasite activity (Eid *et al.*, 1988, *Exp. Parasitol.* **66:** 140) and antiHIV activity (Polsky *et al.*, 1989, *Contraception* **39:** 579). This wide range of biological actions suggests that there might be a parameter common to the cell types affected. In this study, we aim to establish the methodology to identify this common parameter, a protein that is responsible for gossypol binding. We used sperms from marine organisms as a model system because of their various advantages over other cells. Sperm proteins from marine species (*Spisula* and *Arbacia*) were collected and washed with MBL ASW. The sperm pellet was treated with methanol and chloroform to remove lipophilic materials. A protein material was extracted from this lipid-free sperm material by incubation with PBSTDS buffer which contains Triton X-100, deoxycholate, and SDS in phosphate buffered saline. This was incubated with ^{14}C-gossypol (500 μM) for 30 min at 25°C, then a reducing agent, $NaBH_4$, was added. Incorporation of ^{14}C-gossypol was both time and dose dependent. Free gossypol was removed by the chromatography on a Pharmacia Fast Desalting column. Gossypol-binding protein was fractionated by gel filtration on Pharmacia Superose 6; it was eluted with bisTris-EDTA-Tween 20 buffer, at pH 7.5, and at a flow rate of 0.3 ml/min. The protein extract of *Arbacia* sperm contained two fractions with equal amounts of radioactivity: one at the void volume, and the other at 45 min (estimated molecular weight at around 100 kDa). Similar results were obtained from the protein extract of *Spisula* sperm; but in this case, the radioactivity was found predominantly in the second fraction. Analysis on SDS-PAGE showed that fraction 1 gave one major band at 16 kDa with two minor bands at the top, and fraction 2 gave one band at 21 kDa. These results suggest that there are specific proteins modified by gossypol, and these proteins could be a target for gossypol action. The method described here could be used for the isolation of gossypol-binding protein from other cells.

This research was supported by funds from the Rockefeller Foundation.

Mariculture and the Marine Environment

Submersible vehicle observations of deep sea red crabs, Chaceon quinquedens, *off of the U.S. continental shelf.* Robert A. Bullis (University of Pennsylvania, Laboratory for Marine Animal Health, Marine Biological Laboratory).

The purpose of this study was to assess the ability of submersible technology to provide on-site inspection of Crustacea for signs of gross pathology. Concurrently, we sought to discover whether a gradient of shell disease exists as a result of oceanic dispersal of sewage sludge from the Deep Water Municipal Sewage Sludge Dump Site 106. The Harbor Branch Oceanographic Institution submersible *Johnson Sea Link II* was used for *in situ* observations. Rated to a depth of 3000′ (910 m), the *Sea Link* provides unexcelled visibility and maneuverability for observational and experimental studies of midwater benthic communities. Red crabs were monitored for shell disease, and corresponding sediment samples were analyzed for indicator bacteria. Bacterial isolates were successfully obtained from nephloid (surface) sediments at all sampling stations. Greater numbers of bacteria were isolated from shallow (<800 ft.) stations than from deep stations (>2000 ft.). Significant numbers of bacteria, typically associated with shell disease, were isolated from affected, in contrast to non-affected, areas of red crab exoskeletons. Shell disease appeared to occur randomly throughout the population. Of particular note was the observation that, while standing or walking, all red crabs support themselves on the tips of their walking legs. The ventral surfaces of the appendages and the sternum do not scrape the sediment except when the animal is entering or leaving its burrow. Indeed, after the red crabs leave their burrows, nephloid sediments adhere to their carapaces. After a time, sediment remains only in "suture depressions" of the carapace. This prolonged contact between the sediments and certain areas of the exoskeleton may help to explain why hyperpigmentation of red crab carapaces is often bilaterally symmetrical.

This work was sponsored through the generosity of the National Undersea Research Program (NOAA-UCAP) and a grant from the Division of Research Resources, NIH (P40-RR1333-10).

Calibration of the effect of the air-sea interface on measurement of eelgrass and macroalgal density in Waquoit Bay. John T. Finn (University of Massachusetts), Linda A. Deegan, and David Paton.

Waquoit Bay is an important nursery ground for fish and shellfish. Nutrients have caused an increase in macroalgae at the expense of eelgrass beds over the last two years. To examine its effect on eelgrass, we removed macroalgae from one 25 × 25 m plot in an eelgrass bed in Hamblin Pond, a part of Waquoit Bay. The algae removed were added to a second plot, and there were two control plots: one untouched, and one with algae removed and replaced.

Can the amount of algae and eelgrass in the experimental plots—90–120 cm deep—be determined using aerial photography? If aerial photography is to be successful, we must identify the plots from the air and distinguish between the bottom, the macroalgae, and the eelgrass. To test this question several problems had to be addressed. Surface glare was minimized by using a polarizing filter and by taking pictures at times of low sun angle. Ektachrome 100 film was used. Distortion was addressed by placing targets at the plot corners and correcting the photographs to match the true size and shape of the plots. Targets of known color were used to correct for color distortion of dissolved and suspended matter.

To test the feasibility of aerial photography, white locational and color strip targets were photographed in water from 35 to 100 cm deep. Targets disappeared at 55 cm, and colors became indistinct at 45 cm depth. To do the aerial photography, Secchi depth would have to be at least twice what it was during the calibration test. In May or June, when the water is clear, this procedure should allow determination of macroalgal density, although distinguishing between eelgrass and macroalgae would still be a problem.

*Intentional catch (*Busycon*) and unintentional catch (*Hoploplana*) by fishermen and the question of seafood inspection.* ILENE M. KAPLAN, BARBARA C. BOYER, AND DANIELA E. HOFFMANN (Union College, Schenectady, New York 12308).

Data from a longitudinal study on socio-economic and ecological trends in the New England conch (*Busycon*) fishery are reported together with related work on the commensal relationship of the flatworm *Hoploplana*. Special attention was given to marketing conditions and the policies (both formal and informal) that protect these species as well as their consumers. Interviews with fishermen, seafood buyers, processors, and fish market and restaurant owners were conducted, and participant observations were made.

New England conch is different from, but may be confused with, South Atlantic conch (*Strombus*) because they have the same commercial name. Fishermen receive 30 to 50 cents a pound for conch in the shell, which are then sold in New England markets for two to three times their initial worth. Conch meat removed from the shell sells for $3.99 to $4.25 per pound, and in a salad from $3.49 to $5.99 a pound. Customers are typically from Italian and Oriental ethnic groups; foreign export sales include Asian and European markets. The volume of conch sales vary markedly; wholesalers' figures range from 300 pounds to over 8000 pounds per week. Inconsistencies in the reports of sales were found; government reports of sales appear to be more conservative. Variability in the conditions of processing and marketing of conch were also observed. Markets follow inspection guidelines on a volunteer basis, but there are no formal rules for seafood inspection. Although conch is categorized as a shellfish, enforcement of shellfish tagging rules may be variable.

Overfishing of New England conch is a potential problem as commercial interest increases. The number of conch pots has increased dramatically in the last few years, threatening not only the conch, but the flatworm *Hoploplana* that lives commensally in its mantle cavity. A total of 579 specimens of *Busycon canaliculatum* were examined (412 females and 167 males). Twenty-two percent yielded 190 worms. The occurrence of multiple worms per specimen has decreased from previous years, and more worms were found in males than females, also a departure from previous observations.

Formal and unified seafood inspection programs and education and clarification of policies regarding the commercial fishing of conch are suggested.

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Ozonation of natural seawater affects the embryology of Hermissenda crassicornis. ALAN M. KUZIRIAN (Marine Biological Laboratory), CATHERINE T. TAMSE, AND MARK HEATH.

The new MBL Marine Resources Center (MRC) will have three different uses for ozonated seawater: in the recirculating system, in the treated, flow-through system, and in the effluent system. Major aquaria and commercial hatcheries operate at ozone dosages ranging from 0.1 to 2 mg/l. However, the exact dosage appropriate for the new MRC is unknown. We undertook a study to ascertain a preliminary working ozone dosage, using the embryonic development of *Hermissenda crassicornis* as a bioassay.

The following treatment solutions were tested: (1) ozone levels (12.8 min contact time) at 2.2 mg/l, and 0.24–0.28 mg/l; (2) ozone plus 0.1 m*M* Na thiosulfate to remove hypobromic acid; (3) ozone plus NH_3 (0.6–1.2 mg/l), NO_2 (0.05 mg/l) (N-cmpds); and (4) ozone plus thiosulfate and N-cmpds. Controls consisted of 0.2 μ-filtered natural seawater (NSW). Replicates of the solutions, with and without 0.25 mg/l EDTA were used, and each solution was run in duplicate. A single egg mass from *Hermissenda* was cut and distributed equally for each run, which lasted 6 days.

Ozone dosage of 2.2 mg/l resulted in a very unstable ozone residual of <0.1 mg/l; this level should require only minimal aeration for post-treatment. Ammonia concentration did not decrease with ozonation, but all nitrites decreased below detectable limits. Total residual oxidants (TRO), as mg/l Cl_2, registered 0.9 mg/l and slowly decayed to nondetectable limits (70–80 min). TRO for 0.28 mg/l ozone was undetectable.

Bioassay results for 2.2 mg/l ozonation indicated that EDTA or thiosulfate was required for normal development. Ozone plus N-cmpds also produced normal survival rates, but the number of fouling organisms on the shells increased. Ozone with N-cmpds and thiosulfate were deleterious to larval hatch and survival. Dosage of 0.28 mg/l ozone was insufficient to inhibit fouling organisms (diatoms, ciliates); mortality was high for all conditions. Normal development was correlated with the presence of EDTA.

Ozone treatment of MBL seawater is effective at 2.2 mg/l dose levels. Residual ozone and TRO must be removed to insure the survival of sensitive organisms. Biofiltration is supplemented by residual ozone, which oxidizes toxic nitrites to nitrates. The lower ozone dose tested was insufficient as a primary treatment to inhibit the growth of fouling organisms. *Hermissenda* proved to be a sensitive animal for use in this bioassay.

This research was supported in part by a grant to A.M.K. (NIH, RR03820).

Greenough Pond project planning: Yarmouthport, Massachusetts; acid water and macro flora is the base line of H. Sverdop and P. Warfingers' chain of response decision tree. DAVID PATON (BUMP affiliate, Marine Biological Laboratory).

The Greenough kettle pond is spring fed from the unused cranberry bogs at its south west corner. Ground water flows from this direction and includes the Little Greenough Pond. It is one of the more elevated ponds on the Cape and is located near the center of the single source aquifer. This fresh water lens lies beneath a glacial till of sandy soils, sweetened by gravels and dotted with granite boulders, that form the substrata that form the basin of Greenough Pond. The bottom of the pond is silt overlaid with litter from trash that is flown in by seagulls. Milfoil plants dominate the below-surface water weeds, and their growth appears to be depth dependent as they emerge in the shallow coves; they seldom occur below depths of 12′. White lily pads and water shield float on the surface in the quiet water of the west and north coves of the pond.

The pond is acidic. Samples taken in January, April, July, and October, as part of an ongoing monitoring of acid rain deposition taken throughout the Cape, have a typical pH of 5.18. This acidity does not vary more than two tenths of a pH unit over a data set that was compiled from as far back as April 1985. EPA alkalinity is zero or slightly higher.

Halichoerus grypus *at Monomoy Island, winter of 1988–1989: photogrammetry showing shift east and north of traditional birthing sites.* DAVID PATON (Marine Biological Laboratory).

On 13 February 1989, high oblique aerial photographs were taken of the seal habitats in Martha's Vineyard and Nantucket Sounds. This was the second of two flights made that winter which were the only surveys attempted during 1989 to test a behavior prediction model.

High density black and white 70-mm film was used. Positive enlargement transparencies were then projected on a silver screen for viewing. Prints were offered to four biologists for comment. ITEK international Corp. of Bedford, Massachusetts, was consulted for photointerpretive advice. Two of the seal pups appear to have completed the molt from white coat. This represents a shift from the place where these animals have been seen at Nomans Island during the 1930s, and off Nantucket during 1987–1988, and in 1963.

These results are dedicated to the memory of my father. His meteorological career and dedication to education represented his unfailing belief in our stewardship of the land and sea. The Dikenson family supported the winter observations for definition of critical marine habitat.

Neurobiology and Biophysics

Antimalarial drugs block calcium currents in Paramecium. SUSAN R. BARRY (University of Michigan), JUAN BERNAL, AND BARBARA E. EHRLICH.

We studied the effects of antimalarial drugs (quinacrine, chloroquine, and quinine) on calcium currents in *Paramecium calkinsi.* These compounds are structurally similar to W7, a drug that blocks calcium channels in paramecia.

As an initial test of the effects of the antimalarial drugs on calcium currents, we observed the actions of these drugs on calcium-dependent swimming behavior in paramecia. When the paramecium is placed in a high potassium medium (31 m*M* KCl, 94 m*M* NaCl, 1 m*M* CaCl$_2$, and 10 m*M* MOPS, pH 7.3), the cell swims backward for about 50 s. The application of calcium channel blockers, such as W7, reduces the duration of backward swimming. Quinacrine, chloroquine, and quinine all reduced the duration of backward swimming in a concentration-dependent manner. At a concentration of 10 μ*M*, quinacrine inhibited backward swimming by 88%, chloroquine by 37%, and quinine by 29%.

The effects of quinacrine were tested directly on calcium currents using a two-microelectrode voltage clamp. The paramecia were bathed in a sodium-free recording medium containing potassium channel blockers (125 m*M* TEA-Cl, 10 m*M* CsCl, 5 m*M* 4-AP, 5 m*M* 2,4-DAP, 15 m*M* CaCl$_2$, 10 m*M* MOPS), impaled with microelectrodes filled with 300 m*M* cesium citrate, and held at a resting potential of −40 mV. Depolarizing voltage steps evoked an inward calcium current, the peak amplitude of which was reduced by 15% in 10 μ*M* quinacrine, by 51% in 100 μ*M* quinacrine, and by 91% in 1 m*M* quinacrine.

In summary, the effects of quinacrine, chloroquine, and quinine on backward swimming behavior suggest that these drugs inhibit calcium currents. Subsequent voltage clamp studies demonstrated that quinacrine indeed blocks calcium currents. Antimalarial drugs may also reduce calcium currents in other protozoans, including plasmodia, the protozoan parasites that cause malaria. Calcium channel blockade may explain in part the therapeutic effects of these drugs.

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Calcium-dependence of inositol 1,4,5-trisphosphate-gated calcium-channels from endoplasmatic reticulum of cerebellum is bell-shaped. ILYA B. BEZPROZVANNY, JAMES WATRAS, AND BARBARA E. EHRLICH (University of Connecticut Health Center).

Regulation of inositol 1,4,5-trisphosphate (IP$_3$)-induced calcium (Ca) release from intracellular stores has been hypothesized to occur by a direct effect of cytoplasmic Ca on the IP$_3$-receptor. To study the characteristics of this regulation, we incorporated native membrane vesicles from cerebellar endoplasmic reticulum into planar lipid bilayers. Channels were studied with 53 m*M* Ca(OH)$_2$ in 250 m*M* HEPES, pH 7.35 on the *trans* side of the membrane and 250 m*M* HEPES-Tris, 1 m*M* HEEDTA, and 1 m*M* EGTA, pH 7.35, on the *cis* side of the membrane (*cis* corresponds to the cytoplasmic side).

Addition of 2 μ*M* IP$_3$ to the *cis* chamber activated Ca-permeable channels with four conductance states, each a multiple of the lowest conductance (20 pS). Experiments on the Ca-dependence of these channels were performed in the presence 330 μ*M* AMP-PCP, because we found that adenine nucleotides activated the channels about 10 fold. As the free Ca concentration was increased, the probability of observing open channels changed biphasically, with the maximum at about 0.25 μ*M* Ca. This value is in a good agreement with data obtained with IP$_3$-induced Ca-release from permeabilized smooth muscle (Iino, 1990, *J. Gen. Physiol.* **95:** 1103–1122). The Ca-dependence of the channels was very sharp, with half-maximum activation and inhibition at 0.1 μ*M* Ca and 0.5 μ*M* Ca, respectively.

To fit the data with a theoretical curve, we assumed that each subunit of the receptor had two sites and that binding of Ca to one of these sites leads to activation of the channel, and that binding to the other leads to inhibition. We had to assume the existence of a tetrameric structure for the IP$_3$-receptor complex to make the Ca-dependence sharp enough to be in agreement with the data. The existence of the tetrameric structure is supported by four subconductance states observed in our experiments and by electron-microscopic analysis of the purified IP$_3$-receptor.

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Calcium dynamics in the presynaptic terminals of barnacle photoreceptors. JOSEPH C. CALLAWAY (University of North Carolina, Chapel Hill), NECHAMA LASSER-ROSS, ANN E. STUART, AND WILLIAM N. ROSS.

We have measured the time course and magnitude of the [Ca]$_i$ in the presynaptic arbors of individual photoreceptor neurons of the barnacle (*Balanus nubilus*) injected with FURA-2. The ocellus, containing the light-sensitive dendrites, was cut off, and the FURA-filled electrode was used to stimulate the terminal and record membrane potential. The [Ca]$_i$

was recorded at every point along the arbor (about 3 μm resolution), from the primary branches to the spray of smaller processes housing the release sites. Depolarizations with injected current caused a rapid rise in $[Ca]_i$ initially localized to the tips of the spray. The $[Ca]_i$ in proximal parts of the arbor rose more slowly and with a delay as the Ca diffused backwards from the tips to these locations. The threshold voltage for Ca entry was between −55 and −62 mV. During maintained depolarization, Ca entry was continuous and the $[Ca]_i$ at any point in the arbor reached a steady state; as the Ca diffused longitudinally from the release sites into the primary branches, a gradient of $[Ca]_i$ was established. The $[Ca]_i$ at the release sites increased less than 100 n*M* in response to depolarizations that were just above threshold and sufficient to cause transmitter release. The $[Ca]_i$ increased to 400–500 n*M* when the terminals were depolarized to potentials normally achieved by steady light of moderate intensity (−45 mV). When the PR terminal was repolarized from a depolarized level, the $[Ca]_i$ at the tips decreased quickly, as might be expected from this synapse, where *decreases* in transmitter release constitute the important signal. Computer simulations indicate that longitudinal diffusion alone can account for this decrease.

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Cross-correlations in the spike activity of neurons in the Aplysia *abdominal ganglion during the gill-withdrawal reflex.* LARRY COHEN, CHUN XIAO FALK, DAVID SCHIMINOVICH, JIAN-YOUNG WU, AND RAY FALK (Dept. of Physiology, Yale University School of Medicine).

In previous attempts, we were unable to find significant correlations between the spike times of the 100–200 neurons in the *Aplysia* abdominal ganglion that are activated by a light touch to the siphon skin. In these experiments, we weighted the correlation scores inversely to the overall spike density. In this study, we have not made such a correction. We used two methods (raw scores and normalized scores) to evaluate correlations. For the normalized score, the raw score was divided by the number of spikes in the cell of the pair having the smaller number of spikes.

We scored approximately 5000 pairs of cells for each data set. Because the number of pairs and the number of spikes is large, it is not surprising that we detect correlations. To test the possibility that these correlations are due to chance, we generated a random data set from the real data by replacing each spike time with a randomly chosen time point within a 64-ms interval surrounding the real time. We made 10 random sets and compared the correlation scores from the real data with the scores from the 10 random sets. In the trials thus far examined, the correlation scores from the real data were bracketed by the largest and smallest correlation scores from the random data. Thus we did not find correlations larger than might be expected by chance.

If the *Aplysia* nervous system used many large, excitatory synaptic potentials to generate the gill-withdrawal reflex, then we would expect to find larger than random correlations between the activity of cell pairs. Our results suggest that large excitatory synaptic connections are not common. Further, correlation analysis may contribute relatively little to working out the map of synaptic connections among the neurons of the abdominal ganglion.

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Effects of synapsin I on synaptic facilitation at crayfish neuromuscular junction. K. R. DELANEY, Y. YAMAGATA, D. W. TANK, P. GREENGARD, AND R. LLINAS (Marine Biological Laboratory, Woods Hole, MA 02543).

The effects of presynaptically injected phospho- and dephospho-synapsin I on transmitter release were studied in excitatory claw opener junctions in crayfish. We examined release evoked by presynaptic action potentials delivered at 0.5 Hz, which does not produce facilitation, and at higher frequencies (5–50 Hz) where facilitation is prominent. Consistent with previous work on squid giant synapse (Llinas *et al.*, 1985, *PNAS* **82:** 3035) and Mauthner cell synapses (Hacket *et al.*, 1990, *J. Neurophysiol.* **63:** 701), reduction of excitatory junction potentials (EJPs) obtained with 0.5 Hz stimulation was seen 5–30 min after injection. This reduction was coincident with the appearance of Texas red labeled synapsin I in the preterminals contacting the postsynaptic muscle fiber (8 of 9 expts.). The EJP amplitude continued to decline linearly over the course of 60–120 min to near zero. In addition, the rate of facilitation of the EJP during short stimulus trains at 5, 20, and 50 Hz was reduced following this injection. Moreover, high rates of release, which are normally produced and maintained during several minutes of stimulation at frequencies around 30 Hz, were not maintained following dephospho-synapsin I injection. These effects were not seen following the injection of phosphorylated synapsin I (n = 1). We conclude that synapsin I can modulate facilitated and unfacilitated transmitter release at this tonic junction.

Application of a magnetic current probe to map axial inhomogeneities in a squid giant axon. J. M. VAN EGERAAT AND J. P. WIKSWO JR. (Vanderbilt University, Nashville, TN).

The magnetic current probe developed by Wikswo (Gielen *et al.*, 1986, *IEEE Trans. Biomed. Eng.* **BME-33:** 910–921) enables us to measure accurately intracellular action currents in nerve fibers and bundles without the need for physical contact between the probe and the preparation. We exploited this feature to map axial inhomogeneities in the squid giant axon by scanning the probe along the fiber and measuring the action current at many different positions. Similar measurements with intracellular microelectrodes would be hard to perform without damage to the preparation, particularly because a single experiment may involve 250 measurements at 50 different positions.

With our probe, we studied the sequence of events that follows a crush injury to a squid giant axon over a period of 3 h under normal physiological conditions. We observed that the crush blocked propagation and caused a depression of the action currents in the region proximal to the crush. The proximal depression spread retrograde with time. This led to the conclusion that the crushed axon did not seal. Other preparations have been reported to seal and, in addition, to form a partition-like structure in the intracellular space, blocking propagation (Yawo and Kuno, 1985, *J. Neurosc.* **5:** 1626–1632). We simulated this by injecting the axoplasm with a small amount of paraffin oil and verified that our crush measurements never showed any correspondence to the sealed situation. Yawo and Kuno also reported that an increased extracellular calcium concentration contributes to sealing. In the squid giant axon, we observed that a five-fold increase of the extracellular calcium concentration slowed the spreading of the depression but did not lead to complete sealing.

A second case of an axial inhomogeneity was studied in preliminary measurements on axons temporarily exposed to a local cold block. These measurements indicated that the effects of the cold block could be detected in a region that is 3 times larger than the cooled axon segment, even 20 min after removal of the cold block. We are currently investigating whether such effects can be attributed to the block of axonal transport.

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Preliminary optical measurements on the Melibe leonina *buccal ganglion.* CHUN X. FALK, WIN WATSON III,

Jim Trimarchi, Jian-young Wu, and Larry B. Cohen (Dept. of Physiology; Yale University School of Medicine).

Molluscan buccal ganglia have been used extensively to study the neural basis of feeding behavior. However, detailed information about the underlying neural circuits is lacking for most buccal preparations because of their complexity. A previous study, done by Watson *et al.*, (1989, *Soc. Neurosci. Abstr.* **15:** 1139) introduced a relatively simple system, the buccal ganglia in the nudibranch, *Melibe leonina*, which has only about 50 neurons. Their work indicated that the role of the *Melibe* buccal ganglia is to control swallowing. We monitored the activity from the four nerves leaving the ganglion. When the circumesophageal ganglia were removed, the buccal ganglion alone generated rhythmic activity.

We attempted to use optical recording on the *Melibe* buccal ganglion to see if we could measure every action potential in every cell in the ganglion. We did optical recording on preparations stained with a merocyanine dye (JPW1124) or an oxonol dye (NK3041). We did completeness tests on several ganglia. In one ganglion we detected the activity of more than 90% of the cells. In an optical recording done during a rhythm, activity was detected in more than half of the neurons. We achieved a reasonably good signal-to-noise ratio.

By comparing optical and nerve recording, we determined the locations of some cells that have their outputs in the nerves. At this writing, two cells have appeared consistently in all the experiments we have analyzed.

Photo-activation during the optical recording was a serious problem. Photo-activation only occurred in dye-stained preparations. It could be that the dye, alone or together with light, lowers the threshold. We plan to try additional dyes.

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Segmental location of cranial nerve roots and motor nuclei in Squalus acanthias. E. Gilland and R. Baker (Department of Physiology and Biophysics, NYU Medical Center, New York, NY 10016).

Cranial nerve roots III-X were exposed by dissection in paraformaldehyde-fixed embryos of *S. acanthias* between Scammon stages 25 and 30. Crystals of fluorescent lipophilic dyes (DiI and DiA) were applied to the nerve roots in phosphate buffer, and the embryos were incubated at 40°C for 4–12 h. Neuroepithelial wholemounts were cleared in glycerol and viewed with combined epifluorescent-bright field illumination that allowed clear visualization of neuromeres, cranial nerve roots, and motor nuclei. Six interneuromeric borders were observed to delineate rhombomeres (r) 1–7. Sensory and motor roots of nerves V, VII, and IX entered and exited the neuroepithelium of r3, r4, and r7, respectively. The majority of motor neurons contributing to each of these branchiomotor nerves were found at the segmental level of the roots, but in all cases a small number of motor neurons were also located in adjacent rhombomeres (r2 for V, r5 for VII, r6 for IX). At these stages, central sensory tracts of V and VII traversed all hindbrain segments. The motor neurons of nerve X were located posterior to those of IX in a region that lacked any clear segmental demarcation but which may represent an eighth rhombomere. Small clusters of nerve III motor neurons appeared in the mesencephalon, each giving rise to a separate root traceable to a distinct eye-muscle mesenchymal condensation. Motor neurons of nerve IV arose only from r1, and those of nerve VI from four distinct clusters in r6. These data largely confirm Neal's 1918 segmental scheme for the cranial nerve roots and motor neurons of *S. acanthias*. The overall neuromeric pattern of the embryonic shark rhombencephalon is clearly homologous to that of the chick; however, differences in the segmental relations of the roots and motor nuclei of nerves V, VI, VII, and IX suggest that a general vertebrate plan for neuronal organization within a given neuromere cannot be defined at the present time.

The Limulus-*eye view of the world.* Erik Herzog and Robert Barlow Jr. (Syracuse University, NY).

The horseshoe crab, *Limulus polyphemus*, is an excellent model for studying the neural basis of visual behavior. Extensive information exists about the anatomy and physiology of its visual system, and we are beginning to understand the role of vision in the animal's behavior. To relate physiology and behavior, we need to know what the animal sees. Here we examine the eye-world interface by measuring the orientation of the lateral compound eyes in the animal and the lens facets in the eyes.

The orientation of the eye *in situ* is defined by a plane tangential to a marked, central ommatidium. This "eye plane" was determined in two steps with a large, two-dimensional goniometer. First, the animal was rotated about an axis parallel to its horizon and its longitudinal axis, and passing through the ommatidium. Next, a laser was rotated along the horizon about an axis that also passes through the ommatidium until the laser beam reflected back on the face of the laser. The two angles defined the eye plane at the marked ommatidium with respect to the horizon and the animal's longitudinal axis.

The eye was then fixed, excised, mounted in seawater on a second two-dimensional goniometer, and the eye plane was re-established. Axial illumination of the eye reveals a pseudopupil that moves as the eye is rotated. The pseudopupil is composed of a ring of ommatidia that appear dark (because they absorb axial light) surrounding a central ommatidium that glows brightly (because it maximally reflects axial light). The deep aperture of the glowing ommatidium can be centered by rotation of the eye, and the angular settings of the goniometer then define the optic axis of that ommatidium. This procedure was repeated for hundreds of ommatidia in the eyes of five adult males.

The optics of the lateral eye are not homogeneous. The array of lens facets is not regular, and the angles between adjacent ommatidia are not constant. The optical axes of horizontal neighbors diverge most greatly in the ventro-posterior region of the eye (approx. 7.0°). The axes of dorso-anterior ommatidia diverge the least (approx. 3.3°). Ventro-anterior and dorso-ventral ommatidia have intermediate angles of divergence. The optic axes of vertical neighbors diverge 10.4° on average in the dorso-posterior part of the eye, whereas those in the ventro-anterior region diverge the least, at 3.2°. Intermediate divergence (approx. 6°) is found in the other two parts of the eye. Combining all measures of optic axes yields an overall field of view of the eye of about 187° along the azimuth and 87° along the vertical.

In sum, the *Limulus*-eye view of the world is large, especially in the horizontal direction, and has the highest spatial resolution in the forward direction which is the direction of movement.

Research supported by NIH and NSF grants.

Inactivation of squid rhodopsin in the absence of phosphorylation. Alon Kahana, Phyllis R. Robinson, and John E. Lisman (Brandeis University).

Visual transduction involves a cascade of biochemical reactions that are transiently activated by a flash of light. In the first stage of the cascade, light-activated rhodopsin (R*) activates G-protein molecules by catalyzing the exchange of GTP for GDP. Termination of transduction requires inactivation of R*. We monitored R* in suspensions of squid outer segments by measuring light-activated GTPγS binding by G-protein.

Inactivation of R^* is defined as the time-dependent reduction in the rate of light-activated nucleotide binding. Up to 50-fold inactivation occurred in the absence of added ATP or in the presence of kinase inhibitors (AMP-PNP or sangivamycin), indicating that inactivation does not require phosphorylation. Furthermore, added ATP did not significantly enhance inactivation. Further experiments were conducted to test whether inactivation is due to an intramolecular thermal transition of R^* into an inactive state, or to interaction of R^* with another protein. When the retinal suspension was diluted or hypotonically washed, R^* inactivation became minimal. Addition of supernatant back to the washed membranes restored inactivation. These results suggest that inactivation of squid rhodopsin occurs through interaction with a soluble factor. SDS-gel electrophoresis of light and dark suspensions shows a single protein band around 55 kDa that undergoes light-dependent binding to the membranes, making this protein a good candidate for the factor participating in inactivation.

Squid rhodopsin belongs to a family of structurally related transmembrane receptors that includes vertebrate rhodopsin, β-adrenergic and muscarinic receptors. These receptors require phosphorylation for inactivation to occur. Our finding of an ATP-independent inactivation process in squid rhodopsin indicates that phosphorylation subsequent to activation is not a general requirement for inactivation in this receptor family.

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Slices of mouse suprachiasmatic nucleus with attached optic nerve: recording of glutaminergic and GABA-ergic postsynaptic potentials using a voltage-sensitive dye. H. KOMURO, A. L. OBAID, S. S. KUMAR, AND B. M. SALZBERG (University of Pennsylvania School of Medicine).

The suprachiasmatic nucleus of the hypothalamus plays a critical role in the generation and entrainment of circadian rhythms in mammals. Visual input, through the retinohypothalamic tract terminating in the suprachiasmatic nucleus (SCN), participates in the light cycle regulation of circadian rhythms, and ablation of the SCN produces severe disruption or loss of periodicity. However, because of its size, location, and inherent complexity, the properties of the SCN are difficult to study with conventional electrophysiological techniques. Here, we report the use of multiple-site optical recording with voltage-sensitive dyes to facilitate the identification of the important neurotransmitters of the retinohypothalamic pathway to the neurons of the SCN. We used an acute, 350-μm thick, slice preparation from the CD-1 mouse, cut in an oblique plane that included the optic chiasm, one SCN, and the intact optic nerve. After cutting, the slice was rested for at least 1 h in oxygenated mouse Ringer's solution containing 0.003% hydrogen peroxide. The slice was then stained for 30 min in a 100 μg/ml solution of the pyrazo-oxonol dye, RH 155, and changes in transmitted light intensity were measured with a photodiode array, providing millisecond, time-resolved readout of electrical activity from 124 regions of the slice simultaneously. A suction electrode was fitted to the intact optic nerve, and a single 50-μs shock was delivered to the axons of the retinal ganglion cells. The optical signals recorded from the SCN reflect the superposition of numerous postsynaptic membrane potentials. When Ca^{2+} was lowered from 2.2 mM to 0.1 mM, with Mg^{2+} substitution, the amplitude of the signal was dramatically reduced, while raising Ca^{2+} to 7 mM increased it. In the high Ca^{2+} Ringer's solution, a prominent hyperpolarizing phase lasted hundreds of milliseconds.

CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), a potent inhibitor of non-NMDA type glutamate receptors, at 2 μM concentration, completely eliminated the compound postsynaptic potential in 30 min. Kynurenic acid, another blocker of these glutamate receptors, also reduced the amplitude of the signal. These experiments and others suggest that non-NMDA type glutamate receptors play a central role in mediating synaptic transmission in the retinohypothalamic pathway to the SCN.

The long-lasting hyperpolarization, that is particularly evident in high Ca^{2+} Ringer's, might represent the activation of inhibitory pathways within the SCN. Block of $GABA_A$ receptors with 10 μM bicuculline had no effect on the amplitude of the signal, nor on the rising phase, but the width of the signal was greatly increased, as though an inhibitory component of the signal had been reduced or eliminated. Following a 5-min exposure to 1 μM baclofen, a $GABA_B$ agonist, the compound postsynaptic potential was markedly reduced in size. In this case, the activation of $GABA_B$ receptors had its effect very early in the time course of the optical signal, compared with the effect of bicuculline. This suggests that the $GABA_A$ and $GABA_B$ pathways are anatomically distinct, with a longer latency assigned to the bicuculline-sensitive pathway. Baclofen block, unlike the effects of CNQX and bicuculline, was fully reversible.

In summary, we have demonstrated the application of multiple site optical recording techniques to a SCN brain slice preparation having the visual input pathway intact. Also, we have provided physiological evidence for the involvement of a non-NMDA type glutamate receptor in an excitatory pathway, and of both $GABA_A$ and $GABA_B$-mediated inhibitory synapses.

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GABA-induced currents of internal horizontal cells of the skate retina. ROBERT PAUL MALCHOW (Dept. Ophthalmology, University of Illinois College of Medicine, Chicago, IL 60612), RICHARD L. CHAPPELL, PAUL GLYNN, AND HARRIS RIPPS.

Two classes of horizontal cells are morphologically and electrophysiologically distinguishable in the all-rod retina of the skate: the large, distally located external horizontal cells, and the slender, more proximal internal horizontal cells. Previous studies have shown that the external horizontal cells possess an electrogenic transport mechanism for GABA (Malchow, 1989, *Biol. Bull.* **177**: 324). The present experiments were designed to investigate whether internal horizontal cells display a similar uptake system.

Isolated internal horizontal cells from the retinas of *Raja erinacea* and *R. ocellata* were obtained by enzymatic dissociation, and the whole-cell version of the patch clamp technique was used to monitor currents induced by GABA and various agonists applied by pressure ejection from nearby pipettes or by bath superfusion. Pressure ejection of 500 μM GABA elicited an inward current of about 500–750 pA in internal horizontal cells. This current was not mimicked by 1 mM muscimol, a $GABA_A$ agonist, or by 1 mM of the $GABA_B$ agonist (−)baclofen. The current was abolished when cells were bathed in a physiological saline in which all of the sodium had been replaced with lithium, and the response was markedly reduced when the chloride concentration in the saline was lowered from 266 to 16 mM by the substitution of sodium isethionate for sodium chloride. The current induced by GABA persisted when cells were bathed in a physiological saline containing 10 mM 4-aminopyridine, 10 mM cesium chloride, 4 mM cobalt chloride, 25 mM tetraethylammonium, and 1 μM tetrodotoxin, a solution that blocks the majority of voltage-activated conductances. Finally, the GABA-induced current did not reverse into an outward current over the range of −110 to +70 mV.

These findings suggest that internal horizontal cells of the skate retina possess an electrogenic transport mechanism for GABA similar to that present in external horizontal cells.

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Desperately seeking sex neurons: detection of projections in the male sex nerve of Hirudo medicinalis *using nickel and horseradish peroxidase backfills.* MICHAEL NITABACH AND EDUARDO MACAGNO (Columbia University).

Backfills of the male sex nerve of the medicinal leech, *Hirudo medicinalis,* were used to find those neurons in the sixth midbody ganglion that send processes out this nerve. The male sex nerve is a branch of the anterior root of ganglion six that innervates the male sexual organs. For this reason, neurons backfilled from this nerve are good candidates for playing specific roles in the sexual behavior of the leech.

Three dyes were used: 5% biocytin, 30% horseradish peroxidase (HRP), and 5% nickel chloride. Since we already knew that the rostral and lateral penile evertor motor neurons and the Retzius cell send processes out this nerve, the presence of dye in these cells was used as the *sine qua non* for the success of the backfill. With biocytin we often failed to backfill one or more of these cells. With HRP we usually labeled the penile evertor motor neurons, but often failed to label the Retzius cell. This may be because the Retzius cell has a smaller diameter axon in the male sex nerve than the penile evertors. In contrast, in the eight nickel chloride backfills we have tried thus far, both penile evertors and the Retzius cell were invariably labeled. We conclude from this that nickel chloride is the most useful of these dyes for backfilling the male sex nerve of *Hirudo.*

Of course, the purpose of these experiments was to identify new cells that send processes out the male sex nerve. We were quite successful in this respect; we consistently backfilled several neurons that were readily identifiable from experiment to experiment, but that were hitherto unknown. We must now use further anatomical, physiological, and neuroethological methods to elucidate the roles, if any, that these newly identified cells play in the sexual behavior of *Hirudo medicinalis.*

FTX, an HPLC-purified fraction of funnel web spider venom, blocks calcium channels required for normal release in peptidergic nerve terminals of mammals: optical measurements with and without voltage-sensitive dyes. A. L. OBAID, H. KOMURO, S. S. KUMAR, M. SUGIMORI, J.-W. LIN, B. D. CHERKSEY, R. LLINAS, AND B. M. SALZBERG (University of Pennsylvania School of Medicine).

We have been using intrinsic (light scattering) and extrinsic (potentiometric dye absorption) optical signals to study the ionic basis of the action potential in the nerve terminals of the frog neurohypophysis, and the detailed role that Ca^{2+} channels play in the coupling of excitation to secretion. We have already shown (Salzberg *et al.,* 1990, *Biophys. J.* **57:** 305a) that specific channel blocking agents, FTX (Llinas *et al.,* 1989, *PNAS* **86:** 1689) and ω-conotoxin GVIA (Cruz and Olivera, 1986, *J. Biol. Chem.* **261:** 6230), can be used to characterize the Ca^{2+} channels of intact nerve terminals in *Xenopus.* FTX, an HPLC-purified polyamine fraction of funnel web spider venom, and a 385-Da synthetic analog, eliminated the undershoot of the normal action potential and reduced the magnitude of the calcium spike in TTX-TEA pretreated preparations. Both of these effects are indicative of Ca^{2+} channel block. In preparations maximally blocked by 10 μM ω-conotoxin GVIA, the synthetic analogue of FTX exhibited further effect but did not entirely eliminate the active calcium response. Further addition of 200 μM Cd^{2+} left only the passive electrotonus. These results suggest that at least two, and probably three, populations of Ca^{2+} channels are present in the intact nerve terminals of the frog neurohypophysis.

In the mouse neurohypophysis, the S-wave, a component of the light scattering signal that is strongly correlated with peptide secretion, was reduced significantly by dilutions as high as 1:10,000 of the HPLC-purified funnel web spider venom fraction. Neither FTX, nor ω-conotoxin, were able to eliminate completely the S-wave, which could be blocked entirely by 200 μM Cd^{2+}. Our results suggest that, in the mouse neurohypophysis, there are at least two, and possibly three, different populations of Ca^{2+} channels, all involved in secretion.

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Lyoluminescence. G. T. REYNOLDS (Dept. of Physics, Princeton University).

Lyoluminescence (LL) is the term applied to the emission of light when certain materials go into solution. Almost all recent studies have been of LL from irradiated materials. It is natural to think of the phenomenon as a potential technique for dosimetry. Certain organic and inorganic materials, after irradiation, exhibit LL upon dissolution. Certain organics (saccharides, amino acids) closely resemble soft tissue and are therefore of particular interest. In general, the literature to date reports results from very high doses (up to 10^6 rad). Certain irradiated inorganic phosphors (alkali halides) exhibit LL due to the formation of F- and V-centers, which release hydrated electrons upon dissolution. Our work has concerned observations of LL from some of the organics used in liquid scintillators, and from NaCl, with particular interest in detection at doses less than 1 rad. An interesting comparison of reagent grade NaCl and a sample of non-iodized table salt has been made. None of the ingredients ("impurities") of the table salt explains by itself the order of magnitude greater light output over that of the reagent grade.

LL can be enhanced by the addition of selected molecules or ions to the solvent. In the case of NaCl, for example, the addition of 3×10^{-4} M Cu^{++} increases the light output by an order of magnitude (Kalkar and Ramani, 1981, *Radiochem. Radioanal. Lett.* **47:** 203–210). Using such techniques, we have detected doses less than 1 rad with very simple equipment. It is possible to design detection systems permitting measurements of 100 samples at a time, involving multianode photomultipliers, coupled to irradiated samples by tapered light pipes. Also, based on experience with liquid scintillator components, an efficient α detector (for radon) may be possible.

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Calcium channels in identified neurons of Hermissenda crassicornis. EBENEZER YAMOAH, NORMAN STOCKBRIDGE, AND ALAN KUZIRIAN (Marine Biological Laboratory).

We have studied calcium currents in neurons of the pedal ganglion that innervate visceral organs of the nudibranch mollusk, *Hermissenda crassicornis.* The whole-cell patch-clamp technique was used.

The bathing medium consisted of (mM concentrations) 300 choline chloride, 100 tetraethylammonium chloride, 5 4-aminopyridine, 50 magnesium chloride, 10 potassium chloride, 10 HEPES buffer, 10 glucose, and 10 calcium chloride. The pipette solution contained 400 cesium chloride, 50 HEPES, 20 sodium chloride, 10 reduced glutathione, 5 EGTA, 5 MgATP, and 1 GTP. Depolarization from −80 mV produces an inward current that activates at about −30 mV and peaks at about +20 mV. The current inactivates with two time constants. Depolarization from −40 mV also elicits current with a biphasic inactivation. The total current is therefore composed of components with similar activation kinetics, but different inactivation time constants.

Both components of inactivation are voltage dependent. With barium (10 mM) as charge carrier, the fast component of inactivation is unaffected and the slow component is further slowed. Both current components are insensitive to cobalt (600 μM) and blocked by cadmium (300 μM). Lanthanium, however, blocks the slow component fully, but leaves about 50% of the fast component unaffected. The dihydropyridines, nitrendipine (300 μM), and nifedipine (400 μM) selectively inhibit the slowly inactivating component.

These findings suggest the presence of two types of calcium channels with similar activation kinetics, but different inactivation kinetics and pharmacology, in an identified class of neurons of the pedal ganglion of *Hermissenda crassicornis.*

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Physiology and Behavior

Structure of alpha-2 macroglobulin from the horseshoe crab, Limulus polyphemus. Peter B. Armstrong (Laboratory for Cell Biology, Department of Zoology, University of California, Davis, CA 95616-8755), Walter F. Mangel, Atsushi Ikai, Kensal E. Van Holde, and James P. Quigley.

A structural and functional homologue of vertebrate alpha-2-macroglobulin has been identified in the hemolymph and blood cells of the arthropod *Limulus polyphemus* (Quigley and Armstrong, 1985, *J. Biol. Chem.* **260:** 12,715–12,719). The subunit molecular mass, determined by SDS-polyacrylamide gel electrophoresis (reducing conditions), is 185 kDa. The native molecular mass, determined by scanning transmission electron microscopy (STEM) under conditions in which the linear relationship between the STEM large angle detector signal and specimen mass thickness allows the determination of the total molecular mass, was 354 ± 35 kDa. Sedimentation equilibrium measurements gave a value of 366 kDa, independent of solute concentration. Sedimentation velocity experiments indicated a homogeneous component with a frictional ratio of 1.41. Thus, the native structure appears to be a dimer, with a somewhat extended conformation. The behavior during gel permeation chromatography was anomolous, yielding an apparent molecular mass approximately half-way between that expected for the dimeric (370 kDa) and tetrameric (740 kDa) configurations. N-terminal peptide sequence analysis yielded a single sequence, indicating a homo-multimeric structure. Transmission electron microscopy of negatively stained preparations revealed a dimeric butterfly-like structure that collapsed following reaction with chymotrypsin. Gel permeation chromatography also showed a more compact structure for the chymotrypsin-reacted molecule. Reaction with trypsin and plasmin results in multiple products which, when analyzed by reducing SDS-polyacrylamide gel electrophoresis, included two prominant bands of higher molecular mass than the unreacted alpha-2-macroglobulin. Surprisingly these high molecular mass products do not contain covalently bound trypsin.

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Lever-press conditioning in the crab. Green crabs perform well on fixed ratio schedules, but can they count? Richard D. Feinman, Hester Korthals Altes, Sam Kingston, Charles I. Abramson, and Robin R. Forman (State University of New York Health Science Center at Brooklyn).

We previously showed that the green crab, *Carcinus maenas,* could be taught to press a lever if every response was reinforced with food [fixed ratio of 1 schedule (FR 1)] or if every other response was reinforced (FR 2) (Abramson and Feinman, 1990, *Physiol. & Behav.* **48:** 267–272). We have now extended this work to include more protracted schedules of reinforcement. We found that crabs showed high rates of responding on progressively increased fixed ratio schedules, one animal reaching FR 9. Blank controls with an inactive lever (but with food present in the food dispenser) showed much poorer rates of responding but performed well when switched to FR schedules. Cumulative records show that animals frequently paused immediately after reinforcement as seen with vertebrates (on much higher FR schedules). To test whether responses were actually controlled by the number of responses, we subjected a group of four animals to four days of FR 6 training. We followed this with four days of a regimen in which they were rewarded on an FR 6 schedule until three reinforcements had been obtained; they were then switched to extinction (no reward). All animals performed well during acquisition, but only one showed consistent high rates of responding during extinction. Several individual records showed that responses were frequently grouped before reward, but an analysis of intervals revealed this was not a predictable effect. Thus, although crabs are capable of perseverance in fixed ratio schedules, it is unlikely that the numerical value of the required responses enters into the behavior.

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*Search for the biological stimulus of the Cushing response in bluefish (*Pomatomus saltatrix*).* Stephen H. Fox, Christopher S. Ogilvy, and Arthur B. DuBois (John B. Pierce Laboratory, New Haven, CT 06519).

We have found that increasing intracranial pressure (ICP) almost invariably produces an increase of heart rate and blood pressure (BP) in the ventral aorta of bluefish (n = 30). Indeed, BP changes are prompt and almost in direct proportion to the increase of ICP. Cooling the water delays the onset, but does not decrease the magnitude of the BP response. However, oxygenation of the cooled water not only delays the onset, but also reduces the amplitude of the BP response (n = 6). Therefore, cerebral hypoxia seems to be the trigger for the Cushing response in these fish. We then looked for the environmental stimulus that might cause this response in nature. Bluefish can accelerate 3 G, exerting great force between the tail and head. We speculated that the spine might be compressed during swimming, raising ICP. To test this, we produced head to tail compression of the vertebral column in anesthetized bluefish, in dead fish, and in vertebral and cranial preparations, and coupled this with side-to-side flexure, hyperextension, or torsion of the body and spine to simulate fast swimming. These motions, which distorted the shape of the spinal canal, displaced fluid into the head and raised ICP by 20 mm Hg. Next, we measured ICP using a 5-m tube connected to a cranial implant in a bluefish swimming (after recovery from anesthesia) in a 3.7 m diameter round tank at 1.3 m/s. ICP fluctuations of ±20 mm Hg (unrelated to depth) were recorded. Tubing flexion artifact accounted for only ±4 mm Hg of this fluctuation. We conclude that the natural role of this BP response may be to maintain cerebral blood flow during ICP changes produced by swimming at speeds sufficient to cause longitudinal compression which together with lateral or vertical flexion, or torsion of the body, displaces fluid contained in the spinal canal into the head.

Sensory Biology

*Boundary layers and microscale fluid dynamics around the lobster's (*Homarus americanus*) chemosensory appendages.* JELLE ATEMA AND PAUL A. MOORE (Boston University Marine Program, Marine Biological Laboratory).

The three major chemosensory appendages of the lobster, *Homarus americanus*, are the lateral and medial antennules, and the dactyls of the walking legs. Each of these organs contains morphologically distinct sensilla and functions differently. We measured the fluid dynamic properties of the three organs to understand the physical constraints on signal processing resulting from morphological specializations of chemoreceptor organs.

We tested the three organs in a flume with background flow velocities of 0, 3, and 6 cm s^{-1}. Into this flow we injected pulses of 2 m*M* dopamine (DA) from a pipette aimed at the organ from a distance of 4 cm. Pulse duration (valve opening) was always 480 ms. A carbon-filled microelectrode with 30 μm tip diameter was placed against the organ cuticle to detect electrochemically the DA signal in the fluid boundary layer at 5 Hz resolution. The signal rose rapidly in all three organs; the decay, however, was far slower and different for the three organs.

At 0 flow velocity, the rather smoothly structured medial antennule showed smooth exponential decay for 60 s; the hairy walking leg showed initial exponential decay consistently followed by an unexplained slow rise and then a further decay for a total signal duration of 150 s; and the dense tuft of aesthetasc sensilla on the lateral antennule held the signal for over 300 s with a decay profile similar to the dactyl. At flow velocities of 3 and 6 cm s^{-1}, signal durations were about 10 s and surprisingly similar for all three organs. Signal amplitudes ranged from 11 μM in the lateral antennule at 6 cm s^{-1}, to 45 μM in the dactyl at 0 cm s^{-1}. In all organs, flow velocity was inversely correlated with signal amplitude and duration.

The results support the notion that the morphological design of chemoreceptor organs is important for the signal dynamics seen by receptor cells which must sample by molecular diffusion through the organ boundary layer. This is most evident at low flow velocities. Increased flow velocity reduces signal duration and greatly reduces the importance of organ morphology.

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Concentration-dependent tuning in lobster leg chemoreceptor cells. H. F. GERARDO, RAINER VOIGT, AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

Chemoreceptor cells in the walking legs of *Homarus americanus* were studied to determine if their tuning priority (the order of response magnitudes to different compounds) is consistent over varying concentration. Prior determinations of tuning spectra (Johnson *et al.*, 1984, *J. Comp. Physiol.* **155**: 593–604) characterized a cell population by best response 121of each cell to an amino acid at a single, relatively high concentration; *i.e.*, 3.5×10^{-4} *M*. Five amino acids (glutamate, hydroxyproline, betaine, arginine, leucine) and ammonium elicited responses from the greatest number of cells within a population in lobster legs (*ibid.*). In this study, stimulus-response functions to three concentrations (3.5×10^{-4} *M*, 3.5×10^{-5} *M*, and 3.5×10^{-6} *M*) of the same six compounds were measured by extracellular recording. We found four types of response. Type I: response to only one compound; response increases with stimulus intensity. Type II: as I, but response function increases *and* decreases. Type III: response to two or more compounds; responses increase with stimulus intensity, and functions do not cross over. Type IV: as III, but response functions increase, decrease, and cross over. The results are consistent with a model of independent receptor sites for different compounds, and varying mixtures of different sites per cell. In addition, Type II and IV responses imply that stimulus intensity and quality may be co-coded. Type IV responses illustrate concentration-dependent tuning.

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The effect of stimulus duration on the chemoreceptor responses of the medial antennule of Homarus americanus. GEORGE GOMEZ, RAINER VOIGT, AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

The medial antennule of *H. americanus* contains a large population of physiologically well-defined chemoreceptor cells, yet its behavioral function is unknown. Unlike the lateral antennule, it does not flick, nor has it any specialized tufts of receptor sensilla. We investigated its temporal filtering properties in order to compare them with those of the lateral antennules. The medial antennules were excised and immersed in a recording chamber, and hydroxyproline cells were located. These cells were then stimulated with three successive 500 ms pulses of 10^{-4} *M* hydroxyproline at intervals of 15 s. After a 1-min interval, we presented an adapting stimulus of varying length (1, 2, 4, 8 s), followed by another three successive test pulses in 15-s intervals. Response magnitude was measured as the number of extracellularly recorded spikes.

The varying pulse caused a phasic response that lasted less than a second, regardless of the stimulus pulse length. This suggests that, for the medial antennule, stimulus onset, not duration, is an important feature of the chemical signal. Under similar stimulus conditions, receptor cells of the medial antennules generated fewer spikes and adapted faster than cells of the lateral antennules (Voigt and Atema 1988, *Chem. Senses* **13**: 742). In addition, medial cells had not fully recovered after 15 s, because the adapting pulse had a marked effect on responses to subsequent test pulses; thus, stimulus duration affects the disadaptation process.

Supported by NSF (BNS 8812952) to J.A.

Three-dimensional odor flow within the nasal cavity of the bullhead catfish. ADELE PILE, PAUL MOORE, AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

Fluid mechanics is an essential component of chemoreception. To understand the temporal structure of chemical signals encountered by receptor cells, we studied the flow of odor through the olfactory organ of the brown bullhead catfish, *Ictalurus nebulosus*. High resolution nasal casts were made in live catfish using the dental impression material Reprosil. Reprosil is a hydrophobic vinyl polysiloxane that can be cast around the mucus that covers the nasal epithelium. Thus, the casts give an excellent impression of the areas of the nasal cavity that are available for water flow. From these casts and the model of ciliary flow by Doving *et al.* (1977, *Acta Zool.* **58**: 245–255) a model of nasal flow was developed.

Water is drawn into the nasal cavity through the inflow naris by ciliary beating that occurs all along the indifferent epithelium and between the lamellae. This causes the water flow along the raphe. The posterior lamellae are taller and angled, preventing water from directly exiting the outflow naris. The water is then driven between the lamellae to the peripheral ventral edges of the nasal cavity. From here the water proceeds to the outflow naris and exits the cavity.

High resolution electrochemical measurements, deep in the outflow naris, indicated that, at peak, a long lasting chemical signal was diluted

100 times, and short pulses 500 times. However, in both cases the stimulus onset was steep. This was remarkable given the complex manifold of parallel lamellar passages the signal has to travel through. We conclude that the nose of the catfish, an exemplary isomate, is capable of perserving rather accurately the pulse structure of odor plumes.

Supported by NSF (BNS 8812952).

The relationship between flow and chemical signals in providing directional cues for chemically orienting hermit crabs. NAT SCHOLZ, PAUL A. MOORE, AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

This study concerns the movement patterns of chemically orienting hermit crabs (*Pagurus longicarpus*) in four flow conditions (0, 1, 2, and 3 cm/s) and the effect of flow on the structure of the odor signal.

Orientation responses were filmed in a 75 × 120 cm flow-through flume. Homogenized mussel (*Mytilus edulis*) extract was used as a stimulus and constantly released at 50 ml/min from a pipette. To be considered as having given a successful response, a crab had to come within 1 cm of the pipette from a starting point 90 cm distant. Using this criterion, we obtained 85–95% response rates for 70 crabs at each flow rate. Initial observations indicate that tracks become less convoluted as flow rates increase. Whether this results from increased mechanical input, or changes in the structure of the chemical signal, will be determined from high resolution odor analysis. For this purpose, temporal odor profiles were taken with electrochemical electrodes sensitive to dopamine (Moore *et al.*, 1989, *Chem. Senses* **14**(6): 62–74). For directional flow conditions, recordings were made in the center axis of the plume at 15, 45, and 75 cm from the pipette tip. In "still" water, a chemical distribution was established by allowing stimuli to disperse from a nozzle at 20 ml/min for 30 min, and further diffusion for an additional 30 min. Recordings were made at 5, 10, 20, and 30 cm from the nozzle. Preliminary observations show that, for flow conditions, both peak frequency and absolute concentration (peak height) decrease with: (1) distance downstream from the pipette, and (2) decreasing flow speeds. We are continuing to quantify peak parameters to determine the effect of distance and flow velocity on the temporal structure of the chemical signal, and its effect on hermit crab orientation behavior.

Supported by NSF (BNS-8512585).

CONTENTS

REVIEW

Elinson, Richard P., Eugenia M. del Pino, Daniel S. Townsend, Fabián C. Cuesta, and Peter Eichhorn
A practical guide to the developmental biology of terrestrial-breeding frogs 163

BEHAVIOR

Akiyama, T., and M. Yoshida
The nocturnal emergence activity rhythm in the cumacean *Dimorphostylis asiatica* (Crustacea) 178

DEVELOPMENT AND REPRODUCTION

Yoneda, Mitsuki, and Shin-ichi Nemoto
Occurrence of partial nuclei in eggs of the sand dollar, *Clypeaster japonicus* 183

GENERAL BIOLOGY

Alexander, David E.
Drag coefficients of swimming animals: effects of using different reference areas 186

Gunthorpe, M. E., C. S. Sikes, and A. P. Wheeler
Promotion and inhibition of calcium carbonate crystallization *in vitro* by matrix protein from blue crab exoskeleton 191

Shields, Jeffrey D.
Rhizophydium littoreum on the eggs of *Cancer anthonyi*: parasite or saprobe? 201

PHYSIOLOGY

Kingsley, Roni J., Mari Tsuzaki, Norimitsu Watabe, and Gerald L. Mechanic
Collagen in the spicule organic matrix of the gorgonian *Leptogorgia virgulata* 207

RESEARCH NOTE

Lohmann, Kenneth J., Michael Salmon, and Jeanette Wyneken
Functional autonomy of land and sea orientation systems in sea turtle hatchlings 214

ABSTRACTS

Abstracts of papers presented at the General Scientific Meetings of the Marine Biological Laboratory 219

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CONTENTS

No. 1, August 1990

Annual Report of the Marine Biological Laboratory 1

INVITED REVIEW

Pardue, M. L., W. G. Bendena, M. E. Fini, J. C. Garbe, N. C. Hogan, and K. L. Traverse
Hsr-omega, a novel gene encoded by a *Drosophila* heat shock puff 77

BEHAVIOR

Buck, John
Unisex flash controls in dialog fireflies 87

DEVELOPMENT AND REPRODUCTION

Goldberg, Walter M., Ken R. Grange, George T. Taylor, and Alicia L. Zuniga
The structure of sweeper tentacles in the black coral *Antipathes fiordensis* 96

ECOLOGY AND EVOLUTION

Curtis, Lawrence A.
Parasitism and the movements of intertidal gastropod individuals 105
Lonsdale, Darcy J., and Sigrun H. Jonasdottir
Geographic variation in naupliar growth and survival in a harpacticoid copepod 113

GENERAL BIOLOGY

Pennington, J. Timothy, and Richard R. Strathmann
Consequences of the calcite skeletons of planktonic echinoderm larvae for orientation, swimming, and shape 121

PHYSIOLOGY

Dahlhoff, Elizabeth, Sabine Schneidemann, and George N. Somero
Pressure-temperature interactions on M_4-lactate dehydrogenases from hydrothermal vent fishes: evidence for adaptation to elevated temperatures by the zoarcid *Thermarces andersoni*, but not by the bythitid, *Bythites hollisi* 134
Michibata, Hitoshi, Hisayoshi Hirose, Kiyomi Sugiyama, Yukari Ookubo, and Kan Kanamori
Extraction of a vanadium-binding substance (vanadobin) from the blood cells of several ascidian species 140
Shick, J. Malcolm
Diffusion limitation and hyperoxic enhancement of oxygen consumption in zooxanthellate sea anemones, zoanthids, and corals 148

RESEARCH NOTE

Watts, Stephen A., J. Roy, and C. W. Walker
Ornithine decarboxylase exhibits negative thermal modulation in the sea star *Asterias vulgaris*: potential regulatory role during temperature-dependent testicular growth 159

No. 2, October 1990

REVIEW

Elinson, Richard P., Eugenia M. del Pino, Daniel S. Townsend, Fabián C. Cuesta, and Peter Eichhorn
A practical guide to the developmental biology of terrestrial-breeding frogs 163

BEHAVIOR

Akiyama, T., and M. Yoshida
The nocturnal emergence activity rhythm in the cumacean *Dimorphostylis asiatica* (Crustacea) 178

DEVELOPMENT AND REPRODUCTION

Yoneda, Mitsuki, and Shin-ichi Nemoto
Occurrence of partial nuclei in eggs of the sand dollar, *Clypeaster japonicus* 183

GENERAL BIOLOGY

Alexander, David E.
Drag coefficients of swimming animals: effects of using different reference areas 186

Gunthorpe, M. E., C. S. Sikes, and A. P. Wheeler
Promotion and inhibition of calcium carbonate crystallization *in vitro* by matrix protein from blue crab exoskeleton 191
Shields, Jeffrey D.
Rhizophydium littoreum on the eggs of *Cancer anthonyi*: parasite or saprobe? 201

PHYSIOLOGY

Kingsley, Roni J., Mari Tsuzaki, Norimitsu Watabe, and Gerald L. Mechanic
Collagen in the spicule organic matrix of the gorgonian *Leptogorgia virgulata* 207

RESEARCH NOTE

Lohmann, Kenneth J., Michael Salmon, and Jeanette Wyneken
Functional autonomy of land and sea orientation systems in sea turtle hatchlings 214

ABSTRACTS

Abstracts of papers presented at the General Scientific Meetings of the Marine Biological Laboratory 219

No. 3, December 1990

CELL STRUCTURE

Weidner, Earl, Robin M. Overstreet, Bruce Tedeschi, and John Fuseler
Cytokeratin and desmoplakin analogues within an intracellular parasite 237

DEVELOPMENT AND REPRODUCTION

Carroll, David J., and Stephen C. Kempf
Laboratory culture of the aeolid nudibranch *Berghia verrucicornis* (Mollusca, Opisthobranchia): some aspects of its development and life history 243
Komatsu, Miéko, Yasuo T. Kano, and Chitaru Oguro
Development of a true ovoviviparous sea star, *Asterina pseudoexigua pacifica* Hayashi 254
Maruyama, Yoshihiko K.
Roles of the polar cytoplasmic region in meiotic divisions in oocytes of the sea cucumber, *Holothuria leucospilota* 264

ECOLOGY AND EVOLUTION

Amano, Shigetoyo
Self and non-self recognition in a calcareous sponge, *Leucandra abratsbo* 272
Ilan, Micha, and Yossi Loya
Ontogenetic variation in sponge histocompatibility responses 279
Berges, John A., John C. Roff, and James S. Ballantyne
Relationship between body size, growth rate, and maximal enzyme activities in the brine shrimp, *Artemia franciscana* 287
Coon, S. L., M. Walch, W. K. Fitt, R. M. Weiner, and D. B. Bonar
Ammonia induces settlement behavior in oyster larvae 297
Pearce, Christopher M., and Robert E. Scheibling
Induction of metamorphosis of larvae of the green sea urchin, *Strongylocentrotus droebachiensis*, by coralline red algae 304
Rawlings, Timothy A.
Associations between egg capsule morphology and predation among populations of the marine gastropod, *Nucella emarginata* 312

GENERAL BIOLOGY

MacColl, Robert, John Galivan, Donald S. Berns, Zenia Nimec, Deborah Guard-Friar, and David Wagoner
The chromophore and polypeptide composition of *Aplysia* ink 326
McFall-Ngai, Margaret, and Mary K. Montgomery
The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda:Sepiolidae) 332

PHYSIOLOGY

Bowlby, Mark R., Edith A. Widder, and James F. Case
Patterns of stimulated bioluminescence in two pyrosomes (Tunicata: Pyrosomatidae) 340
Kallen, Janine L., S. L. Abrahamse, and F. Van Herp
Circadian rhythmicity of the crustacean hyperglycemic hormone (CHH) in the hemolymph of the crayfish 351
Kuhns, William J., Gradimir Misevic, and Max M. Burger
Biochemical and functional effects of sulfate restriction in the marine sponge, *Microciona prolifera* . . . 358
Toulmond, Andre, Fouzia el Idrissi Slitine, Jacques de Frescheville, and Claude Jouin
Extracellular hemoglobins of hydrothermal vent annelids: structural and functional characteristics in three alvinellid species 366
Trapido-Rosenthal, Henry G., Richard A. Gleeson, and William E. S. Carr
The efflux of amino acids from the olfactory organ of the spiny lobster: biochemical measurements and physiological effects 374

Index to Volume 179 383

Erratum

The Biological Bulletin, Volume 179, Number 1, page 91

The following correction should be made in the article by John Buck, titled "Unisex flash controls in dialog fireflies" (*Biol. Bull.* **179:** 87–95). The words "found to be" were deleted from the sentence beginning on line 17 in the right hand column of page 91. The sentence should now read: "By programmed stimulation, the triggering was found to be confined to the latter half of the flashing cycle . . ."

Reference: *Biol. Bull.* **179:** 237–242. (December, 1990)

Cytokeratin and Desmoplakin Analogues within an Intracellular Parasite

EARL WEIDNER[1], ROBIN M. OVERSTREET[2], BRUCE TEDESCHI[3],
AND JOHN FUSELER[4]

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Abstract. A significant amount of the total protein in the spore sacs of the microsporidian *Thelohania* sp. consisted of the cytoskeletal elements, cytokeratin intermediate filaments, and the desmosomal analogues. The cytokeratin and desmosomal analogues were organized as cage envelopes surrounding the spores within the spore sac stage. *Thelohania* sp. parasitizes the skeletal muscle of *Callinectes sapidus*, a crustacean that does not appear to have cytokeratins or desmosomes. Immunoprobe data indicate *Thelohania* sp. has a 240 kDa desmoplakin protein and 48, 51, 54 and 56 kDa cytokeratin polypeptides responsive to antibodies developed against bovine cytoskeletal counterparts. The cytoskeletal envelopes within the *Thelohania* sp. spore sac stage appear to enhance the stability and viability of the spores.

Introduction

Cytokeratin intermediate filaments and desmosomal proteins are specific markers for epithelial cells in vertebrates (Cowin *et al.*, 1985; Romano *et al.*, 1986). Using high resolution two-dimensional gel electrophoresis, over 15 distinct cytokeratin polypeptides have been characterized from vertebrates (Cooper *et al.*, 1984). Monoclonal antibody studies indicate that some of these cytokeratins have analogues present in lower vertebrates (Rungger-Brandle *et al.*, 1989). Indeed, antibody cross-reactivity studies indicate that intermediate filament (IF) epitopes are shared among the different IF families; this cross-reactivity extends to IFs present in many invertebrate groups (Bartnik and Weber, 1989). However, there is no evidence that a monoclonal antibody, directed to mammalian cytokeratin, cross-reacts with presumptive cytokeratin counterparts present in epithelial cells of invertebrates (Fuchs and Marchuk, 1983; Weidner, unpub. data).

The desmosomal plaque elements consist of desmoplakin I (240–250 kDa), a protein that localizes to the region of the desmosomal plaque where cytokeratin binds (Jones and Goldman, 1985). Desmoplakin antibody shows cross-reactivity to this protein in epithelia from various vertebrate groups (Rungger-Brandle *et al.*, 1989). While desmosome assemblages and cytokeratin-like IFs are apparent in invertebrate epithelial cells, these proteins are reported to be absent in arthropods (Bartnik and Weber, 1989). The absence of these proteins in arthropods is noteworthy because we report here that cytokeratin and desmoplakin analogues are present in an intracellular parasite found within arthropods. The cytokeratin and desmoplakin analogues present in the microsporidian parasites cross-react with monoclonal antibodies directed to mammalian cytokeratins and desmoplakin.

Materials and Methods

Animal and cell preparations

Thelohania sp. was taken from blue crabs (*Callinectes sapidus*) collected from Mississippi Sound and the west coast of Florida near St. Petersburg. After removing the *Thelohania* sp.-infected muscle, the dissociated infected muscle fibers were applied to glass slides and fixed, permeabilized in 100% methanol, and further processed for immunofluorescence microscopy. Other infected muscle was washed in 0.5 mM $CaCl_2$, and the *Thelohania* sp. spore sac stage was liberated and purified into populations

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of spore sacs following a wash cycle described elsewhere (Weidner, 1976).

Electron microscopy

Infected skeletal muscle with *Thelohania* sp. spore sac stages from the blue crab, *Callinectes sapidus*, were prepared so that the stages of spore sac development could be examined. Also, isolated spore sacs were fixed, washed, embedded, and processed for electron microscopy as described elsewhere (Overstreet and Weidner, 1974).

Antibodies

The following primary antibodies were used: mouse cytokeratin antibody clones Lu5, AE1 and 3 (Boehringer Mannheim, Indianapolis, Indiana), K8.12, K8.13, DK802 (Sigma Chemical Co., St. Louis, Missouri), and mouse desmoplakin antibody clones PD2.15 and PD2.17 (ICN, Costa Mesa, California). Whereas, clones K8.12, K8.13, Lu5 and AE1 and 3 react to a number of epitopes common to cytokeratins, DK802 has affinity for desmosomal-binding cytokeratin 8. Second antibodies were rabbit immunoglobulins against mouse immunoglobulins coupled to alkaline phosphatase, FITC, or peroxidase (Sigma Chemical).

Immunofluorescence and immuno-electron microscopy

Infected blue crab muscle fibers or isolated *Thelohania* sp. spore sacs were fixed and permeabilized in 100% methanol and processed for indirect immunofluorescence as described elsewhere (Pasdar and Nelson, 1988). Cells were washed in PBS and incubated with anti-desmoplakin or anti-cytokeratin diluted 1:100 with PBS for 30 min at 37°C. After five washes in PBS, cells labeled with anti-mouse immunoglobulin coupled to FITC (Sigma Chemical). After five washes with PBS, cells were mounted in 20% glycerol and viewed with a 60× objective on a Nikon Microphot FXA equipped with epifluorescence illumination; images were recorded on Tri-X film (Eastman Kodak, Rochester, New York). For immuno-electron microscopy, cells were permeabilized with methanol and further fixed in 1% glutaraldehyde for 20 min. Cells were then washed in cacodylate buffer, transferred to PBS, and later immersed into primary antibody in PBS for 1 h. After 30 min of repeated washings in PBS, cells were exposed to anti-mouse conjugated to peroxidase (Sigma Chemical) for 30 min, washed in PBS, and exposed to diaminobenzidine working medium and processed for electron microscopy as described earlier (Pleshinger and Weidner, 1985).

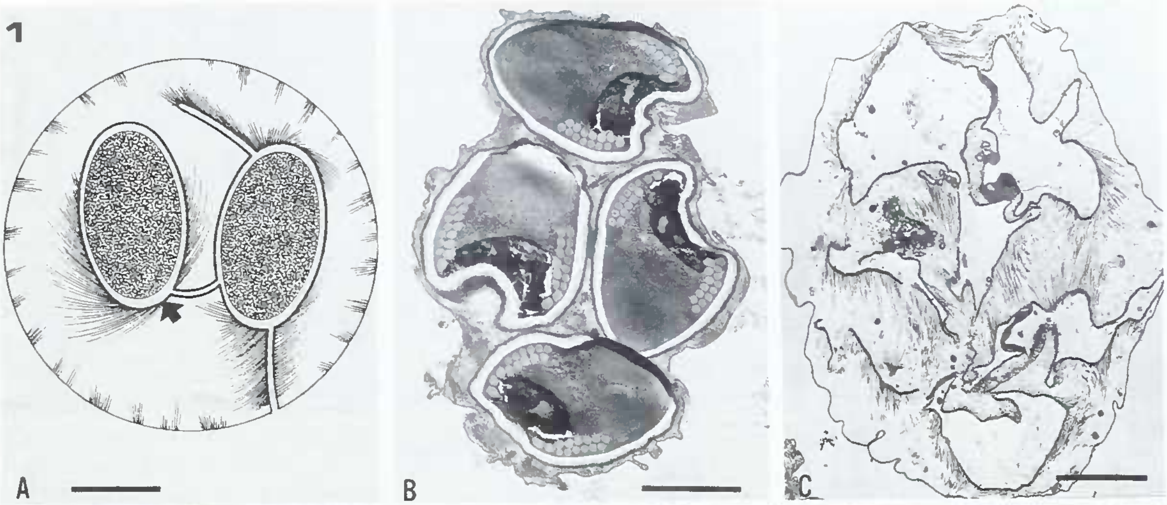
Gel electrophoresis of Thelohania *sp. spore sac proteins and immunoblotting*

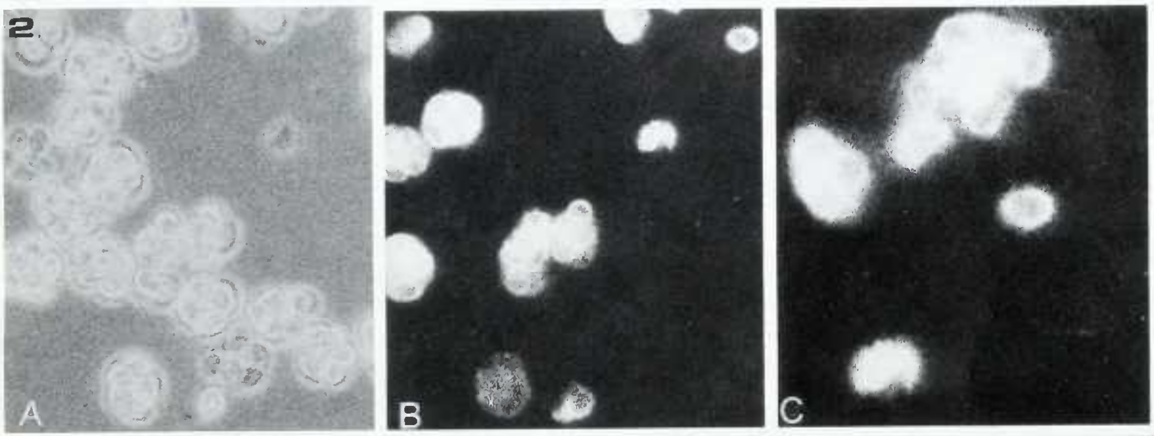
Spore sacs were liberated from *Thelohania* sp.-infected blue crab muscle with a glass homogenizer. The cell suspension was placed in 0.5 mM $CaCl_2$ washed and processed to purification with the wash cycle described earlier (Weidner, 1976). The spore sac desmosomal analogues were disrupted by immersing the sacs in 0.5 mM EGTA for 30 min. Protein samples were prepared by homogenization in boiling SDS sample buffer. The discontinuous buffer system of Laemmli (1970) was used in polyacrylamide gradient gels (7.5–15%). Samples with 15–20 μg protein per lane were heated for 5 min with 2% SDS and 3% 2-mercaptoethanol before loading for electrophoresis. Proteins were stained with Coomassie blue. Proteins for duplicate unstained gels were transferred electrophoretically to nitrocellulose membrane using a Bio-Rad trans-blot apparatus (Bio-Rad Laboratories, Palo Alto, California) overnight at 4°C 50V in Tris-glycine buffer, pH 7.5 with 20% methanol. The nitrocellulose was treated with blocking buffer (1% milk powder, 0.02% Tween 20, 0.02% sodium azide in PBS) for 3 h before incubating in primary antibody (1:100 dilution in blocking buffer overnight). The nitrocellulose membrane was washed five times (10 min/wash) in PBS and transferred to anti-mouse coupled to alkaline phosphatase (1:100 dilution in blocking buffer) for 6 h. The nitrocellulose membrane was then washed five times (10 min/wash) in PBS and the antibody was visualized in incubating nitrocellulose in alkaline phosphatase substrate (100 mM Tris, pH 8.8, 0.01% nitroblue tetrazolium and 0.005% 5 bromo-4-chloro-3-indolyl phosphate) (Sigma Chemical).

Results

A large percentage of the microsporidian parasites develop a sporophorous vesicle (spore sac) stage that surrounds the spores. Within *Thelohania* sp., the extrasporular space within the spore sac stage is filled with intermediate filaments (IFs) that appear attached to desmosomal or half-desmosomal plaques as illustrated in Figure 1A. Within *Thelohania* sp., eight spores are tightly packed within a spore sac (Fig. 1B). The spore sacs were extremely stable and resisted dissociation in dithiothreitol, 2-mercaptoethanol, SDS, 10 M urea, methanol, or organic solvents such as choloroform. However, *Thelohania* sp. spore sacs were partially permeabilized with 0.5 EGTA and subsequent shearing with a glass homogenizer caused up to 20% of the spores to liberate from the spore sacs as shown in Figure 1C.

Fluorescent antibody labeling for cytokeratins and desmosomal proteins showed a strong fluorescence primarily on the spore envelopes of *Thelohania* sp. Figure 2A depicts phase optical imaging of spore sacs recovered from infected muscle of *Callinectes sapidus*. On the basis of antibody labeling, only 15–20% of the spore sacs were successfully permeabilized for antibody labeling. All spores liberated from the sacs were reactive to specific antibodies





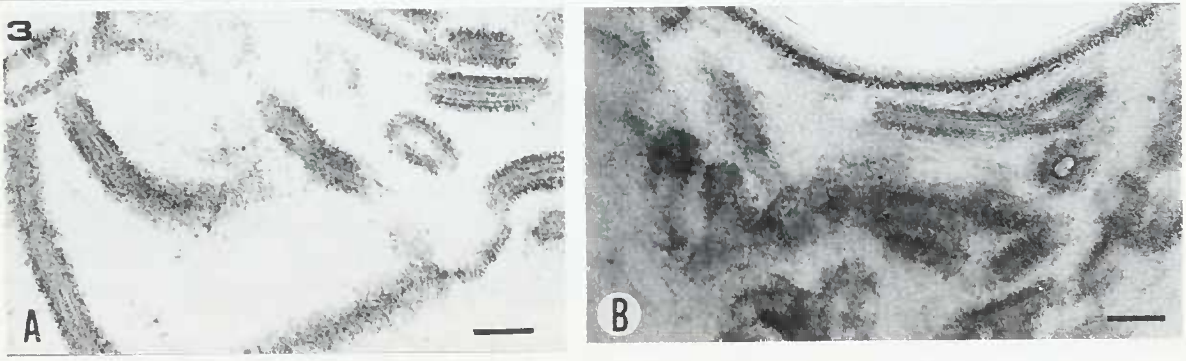


Figure 1. Low magnification images of *Thelohania* sp. spore sacs. Figure A is a diagram of a spore sac with spores. Note the arrow indicating a plaque envelope bearing cytokeratin analogues surrounding spores; plaque surrounding spores appear as half-desmosomes while plaque connecting spores appear as desmosomes. Figure B is an electron micrograph of a spore sac with spores. Figure C is an electron micrograph of a spore sac with plaque envelopes but without spores. Note the IF-bearing half-desmosome plaques that originally enveloped spores in Figure C appear like those illustrated in diagram in Figure A. Bar = 1 μm.

Figure 2. Light microscopy images of *Thelohania* sp. spore sacs. Figures A and B are phase and immunofluorescence imaging of the same field of spore sacs. About 30% of the spore sacs of *Thelohania* in Figure B were permeabilized adequately to enable anti-cytokeratin binding; all liberated spores were positive for cytokeratins. Figure C is an enlargement of Figure B; note that the fluorescence of individual cytokeratin bundles produces a fuzzy impression at the spore surface. The single spores in Figure C are 4 μm in length.

Figure 3. Electron micrographs of desmosomes within a *Thelohania* spore sac. Figure A is a lead-stained image of desmosomes. Figure B shows desmosome plaques immunoperoxidase-labeled for desmoplakin; there is no staining of the cytokeratins. Bar = 50 nm.

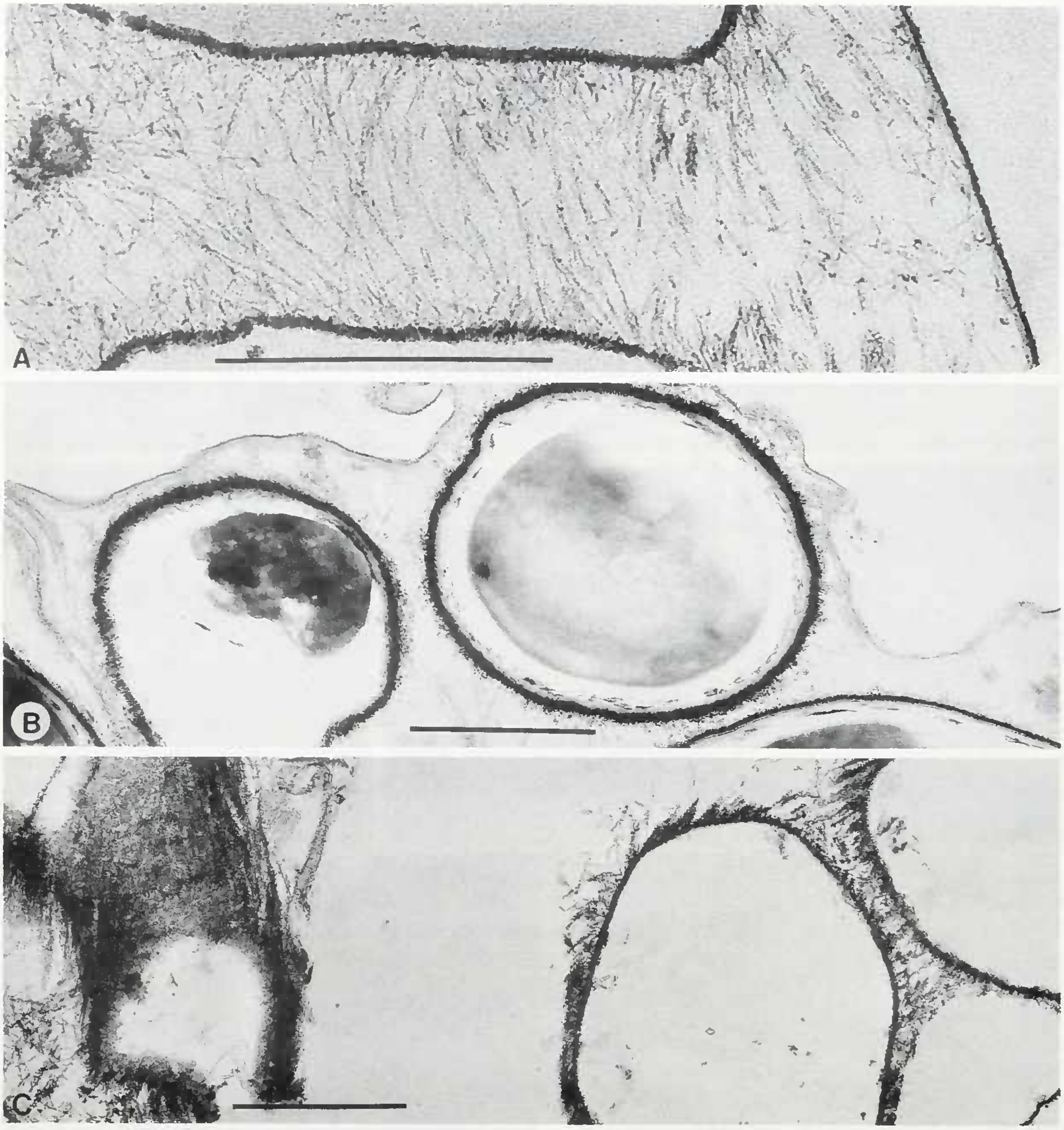


Figure 4. Electron micrographs of *Thelohania* sp. spore sacs. Figure A shows a portion of spore sac without spores. Note the half-desmosome plaques with cytokeratin analogues attached. Figure B shows the half-desmosome plaques with immunoperoxidase labeling for desmoplakin I; note that the cytokeratin analogues within the spore sac appear unstained. Figure C shows a contrasting image of half-desmosomal plaques immunoperoxidase stained for cytokeratins; note that the filaments stand out much more clearly than observed in Figure B. Bar = 0.5 μm.

for cytokeratins or for the desmosomal constituent, desmoplakin. Figure 2B shows fluorescent antibody activity for cytokeratins in field of spore sacs viewed in Figure 2A with phase optics. Figure 2C shows an enlargement of a few fluorescent antibody-labeled *Thelohania* sp. spores. The hairy surface of fluorescence at the spore surface is attributed to the spore-bound IFs. Both desmoplakin and cytokeratin antibody activities were confined to the *Thelohania* sp. spore sacs throughout the different stagings in microsporidian development; thus, host muscle tissue domains were negative for cytokeratin and desmoplakin analogues.

Ultrastructure of *Thelohania* sp. spore sacs showed an abundance of cytokeratin IFs attached to half-desmosome-like plaques enveloping the spores (Fig. 4A). The half-desmosomal plaques that envelope each spore join to form desmosome-like attachments between the spores (Fig. 1A, Fig. 3A). Immunostaining with peroxidase conjugate directed to desmosomal indicator, desmoplakin 1, yielded peroxidase staining on both the desmosome-like structures (Fig. 3B) and the half-desmosomal analogues surrounding the spores within the *Thelohania* sp. spore sacs (Fig. 4B). Immuno-localization of antibody-peroxidase for cytokeratin analogues revealed obvious staining for bundles of IFs bound to the plaques enveloping the microsporidian spores (Fig. 4C).

For further analyses of the number of cytokeratin analogues reactive to keratin antibodies, proteins recovered from purified *Thelohania* sp. spore sacs were subjected to gel electrophoresis and immunoblotting. Monoclonal antibodies AE1 and AE3, K8.13, and Lu5 reacted to epitopes common to cytokeratin analogues from *Thelohania* sp. spore sacs. Immunoblots show response bands near positions 50, 54, and 56 kDa (Fig. 5A). Monoclonal antibody K8.12 reacted to two bands, indicating the presence of cytokeratin analogues 13 and 16 (51 and 48 kDa). For identifying desmosomal proteins, monoclonal antibodies DP2.15 and DP2.17 were used. These antibodies were responsive to a single 240 kDa band corresponding to desmoplakin 1 in *Thelohania* sp. spore sacs (Fig. 5B). In the controls, DP2.15 antibody responded only to the 240 kDa desmoplakin band from bird (turkey) wing tegument (Fig. 5C).

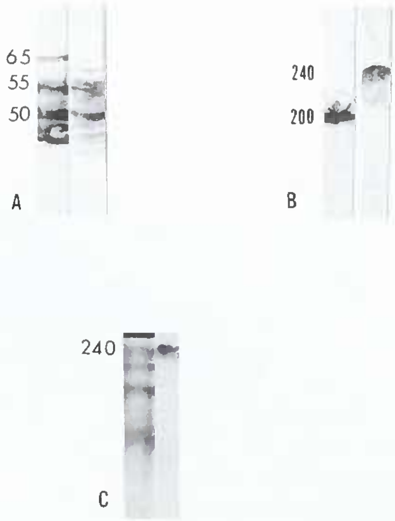


Figure 5. Immunoblot analysis of *Thelohania* sp. spore sac proteins. (A) Lane one shows standard cytokeratins resolved (from human epidermis) into bands in the 65, 58, 56 and 50 kDa range. Lane 2 shows distinct bands of 56, 54, 52, 50 and 48 kDa for cytokeratins from *Thelohania* spore sacs. (B) Lane 2 shows a 240 kDa response band for desmoplakin I; lane 1 shows the molecular weight marker myosin (200 kDa). (C) Control showing desmoplakin I. Lane 1 shows stained proteins from turkey epidermis. Lane 2 shows an immunoblot of lane 1 with a response band to desmoplakin I.

Discussion

The results of this study indicate that cytokeratin IFs and desmosomal proteins appear to be expressed within the microsporidian spore sac stages found within the skeletal muscle of the crustacean, *Callinectes sapidus*. Monoclonal antibody labeling, applied to immunofluorescence and immuno-electron microscopy, indicate an abundance of cytokeratin IFs and desmoplakin analogues within *Thelohania* sp. spore sacs. Curiously, these proteins are reported to be absent in arthropods (Bartnik and Weber, 1989). Immunolabeling data, supported by gel electrophoretic and immunoblot analyses, indicate that the cytokeratin and desmoplakin analogues recovered from the microsporidian spore sacs were immunologically responsive to antibodies prepared against bovine cytoskeletal counterparts. This is surprising because cytokeratins have diversified rather significantly among vertebrates. Antibodies directed to bovine cytokeratins would not be expected to respond to cytokeratin analogues from a lower eukaryote (Fuchs and Marchuk, 1983). Additionally, it was unexpected to find cytokeratin IFs in blue crab skeletal muscle because neither skeletal muscle nor arthropods appear to express cytokeratins. However, the cytokeratins and desmoplakin analogues were confined to the microsporidian spore sac domains within the skeletal muscle.

Two major lines of evidence indicate that the spore sac structure represents a stage in microsporidian life cycles. First, the majority of microsporidian species have spore sacs as a stage in their life cycle (Canning *et al.*, 1982; Becnel *et al.*, 1986). Second, during microsporidian spore sac development, the spores differentiate internally within a progenitor cell in which the extrasporular cytoplasmic domain becomes the spore sac (Overstreet and Weidner, 1974).

The binding of cytokeratin analogues to desmoplakin-bearing plaques in *Thelohania* sp. spore sacs resembles the cytokeratin IF attachments in vertebrate tegumental epitheilium (Kelly, 1966). However, the binding patterns differ because cytokeratin in epithelial cells binds only to membrane after it is stabilized by attachments to adjoining membrane from another cell. In *Thelohania* sp., however, the cytokeratin and desmoplakin plaque binding is primarily to that membrane which is attached to spore surfaces. Thus, cytokeratin and plaque analogues in *Thelohania* spore sacs bind to membrane that has firm attachments to spore surfaces.

The identification of cytokeratin polypeptides in *Thelohania* sp. spore sacs is very preliminary; nevertheless, cytokeratin 13 and 16 appear to be present because 51 and 48 kDa proteins respond to monoclonal antibody K8.12. Also, there is some evidence of cytokeratin 8 (desmosomal cytokeratin), a protein which has a 52 kDa molecular weight.

The origins of desmoplakin-cytokeratin IF expression in Thelohania *sp.*

It is unlikely that IF and desmosomal cytoskeletal genes are native to the microsporidian species in general because only a small percentage of the microsporidians express these proteins. Furthermore, it is unlikely that the microsporidians acquired the capabilities for expressing these proteins from arthropod hosts because these animals do not appear to express the cytokeratins or desmosomes (Bartnik and Weber, 1989). However, it would seem more likely that *Thelohania* sp. may have acquired the cytokeratin and desmosomal genes from a vertebrate source. This is within the realm of possibility because nearly all microsporidians begin growth in epithelial cell lines; and, nearly 100 species of microsporidians have been reported parasitizing aquatic vertebrate animals (Canning and Lom, 1986).

Acknowledgments

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Laboratory Culture of the Aeolid Nudibranch *Berghia verrucicornis* (Mollusca, Opisthobranchia): Some Aspects of Its Development and Life History

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Abstract. Adult *Berghia verrucicornis* individuals lay white, spiral egg masses containing zygotes. Egg masses are easily cultured in aerated, Millipore-filtered, seasoned aquarium water. Development proceeds quickly, with the bilobed velum apparent by the end of the second day, and the larval shell appearing at the beginning of the third day after oviposition. Hatching occurs 11 to 12 days after oviposition (23.9 ± 1.3°C). If egg masses are incubated without aeration, poecilogonous development is observed; both larvae and juveniles hatch from the same undisturbed egg mass. The larvae metamorphose soon after hatching, losing the velum and larval shell. A habitat-specific inducer is not required for metamorphosis; but a factor associated with the sea anemone *Aiptasia pallida* appears to enhance a larva's tendency to metamorphose. Juveniles begin feeding on *A. pallida* three to four days after metamorphosis. Reproductive maturity is achieved as early as 47 days after oviposition. Because *B. verrucicornis* can be cultured, along with its prey *A. pallida*, at inland facilities, this nudibranch species may be a useful model for laboratory-oriented life history and neurobiological investigations.

Introduction

Successful opisthobranch culture has been limited to species with life histories that tie them to fresh seawater. Among the limiting factors have been: (1) a planktotrophic larval stage that requires long-term culture best accomplished with fresh, natural seawater, or (2) a prey species for both juveniles and adults that cannot be easily cultured in sufficient quantity in the laboratory (Kriegstein *et al.*, 1974; Harris, 1975; Kempf and Willows, 1977; Switzer-Dunlap and Hadfield, 1977; Bickell and Kempf, 1983; Paige, 1988). Thus, an opisthobranch species that could be reliably cultured through successive generations in a laboratory environment lacking ready access to fresh seawater would be a valuable source of developmental stages, juveniles, and adults for research in such diverse areas as behavior, development, and ecology (*e.g.*, Bonar and Hadfield, 1974; Kandel, 1979; Todd, 1981; Marcus *et al.*, 1988; Kempf, 1989a; Kempf and Todd, 1989). In particular, opisthobranch mollusks have become premier models for neurobiological investigations, because neurons in their central nervous system are large, repeatably identifiable, and easily manipulated (Willows, 1971, 1973; Kriegstein, 1977a, b; Hening *et al.*, 1979; Jacob, 1984; Kempf *et al.*, 1987; Cash and Carew, 1989; Chia and Koss, 1989; Marois and Carew, 1989).

We selected the aeolid nudibranch *Berghia verrucicornis* as a likely candidate for successful laboratory culture. This mollusk occurs on coral rubble in the shallow waters of southern Florida and feeds on the common anemone *Aiptasia pallida*. The adult attains lengths of 2–3 cm, and its dorsum is covered with cerata that appear brown when the nudibranch has been feeding. Among the characteristics that anticipate the successful culture of this species in laboratories lacking a ready supply of fresh sea water are: (1) a short generation time (adulthood is reached five to six weeks after oviposition), (2) lecithotrophic larvae that undergo metamorphosis within days of hatching from the egg mass, and (3) a juve-

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Abbreviations: MFSA = Millipore-filtered, seasoned, aquarium water; MFSA + A = MFSA plus the sea anemone *Aiptasia pallida*; MFSA-A = MFSA from aquaria containing *A. pallida*, AFSA = *Aiptasia*-free, seasoned, aquarium water; MFSA from aquaria that had never contained *A. pallida*; BF = bacterial film culture.

nile and adult prey that can also be reared in the laboratory.

We suggest that *Berghia verrucicornis* could be a useful opisthobranch species for investigators at inland, as well as marine facilities. Herein we describe the culture techniques used to rear and maintain this nudibranch in the laboratory and give an overview of embryonic and larval development. Certain life history traits, such as: (1) induction of metamorphosis; and (2) the potential for developmental variability in this species (poecilogony), are also discussed.

Materials and Methods

Collection of adult animals

Adult individuals of *Berghia verrucicornis* were collected in southern Florida, in abandoned coral quarries on Grassy Key and near Bahia Honda Key, at depths of less than 6 feet during late December 1987, 1988, and March 1988, 1989. They were transported to Auburn, Alabama in aerated buckets of seawater with fresh seawater changes every 4–5 h. The sea anemone *Aiptasia pallida* was also collected in the Florida Keys and transported to Auburn University as food for the juvenile and adult *B. verrucicornis.*

Culture of the sea anemone Aiptasia pallida

Aiptasia pallida may be cultivated in typical salt-water aquaria with undergravel filtration; wet/dry trickle filters should also be adequate. Our own system consists of a number of individual aquaria, as well as a large-scale culture system consisting of one 110-gallon, four 30-gallon, and two 20-gallon aquaria connected together with flow-through water circulation. One week after set-up, new aquaria with undergravel filtration are conditioned by the addition to each of a few small salt-water fish (*e.g.*, clownfish or damsel fish) or invertebrates (*e.g.*, hermit crabs, anemones). These animals are fed and maintained for at least one month; their metabolic and digestive wastes provide for the growth of essential, gravel associated, bacterial populations that detoxify ammonia and nitrites.

At the end of the conditioning period, a number of *Aiptasia pallida* (20–30) are added to each aquarium. Anemones may be obtained from the field or from various suppliers such as Carolina Biological Supply Co. The anemones are maintained under a combination of "Grow-Lux" and Actinic Blue fluorescent lighting and are fed newly hatched brine shrimp every two days. With appropriate care, the number of *A. pallida* will gradually increase as clones develop from pedal laceration. Regular replenishment of aquarium water with freshly prepared, artificial seawater is important. Individual aquaria are most easily replenished each day, when "seasoned aquarium water" is removed for use in the culture of egg masses, larvae, juveniles, and adult *Berghia verrucicornis* (Fig. 1A, B, C). Healthy anemone colonies should be established before an attempt is made to establish a colony of *B. verrucicornis.* Other, more detailed, methods for culture of *Aiptasia* have been described by Hessinger and Hessinger (1981).

Culture and feeding methods for Berghia verrucicornis

Figure 1 summarizes the culture methods used. Pairs of adult *Berghia verrucicornis* used for egg mass production were kept in glass bowls containing 300–350 ml of 0.45 μm Millipore-filtered artificial seawater (Instant Ocean or Tropic Marine Systems, Inc.) that was obtained from established saltwater aquaria supplied with $CaCO_3$ gravel and undergravel filtration. This water is designated as "Millipore-filtered, seasoned, aquarium water" (MFSA). The water and bowl were changed daily for each culture. As opisthobranch egg masses were layed, they were transferred either to aerated 500-ml beakers containing 350 ml of MFSA or, in the case of experiments concerned with direct development, to unaerated 300-ml glass crystallizing dishes containing 100 ml of MFSA. Water and containers were changed daily. Harvested *Aiptasia pallida,* used for food, were also kept in bowls of MFSA; both bowls and water were changed every few days.

Two days before the expected date of hatching, two or three small *Aiptasia pallida* were placed in fresh dishes containing MFSA. The MFSA in these cultures was changed each day. When the larvae hatched, we concentrated them by pouring the culture water through a Nitex strainer (see Switzer-Dunlap and Hadfield, 1981); we then pipetted them into the dishes containing anemones, taking care to release them underwater so that they would not be trapped at the air-water interface. The number of larvae in each metamorphosis dish varied depending upon the experiment.

Metamorphosis usually occurred in one to three days, and crawling juveniles were present by the third day. After a successful metamorphosis, and until the nudibranchs began feeding, the culture water was changed on alternate days. Thereafter, sea anemones were added as needed, and the culture water and container were changed daily. See Figure 1 for additional details about the frequency with which the water and culture containers may be changed for early juveniles.

Antibiotics were not used in any stage of culture. Regular water changes were sufficient to prevent problems with protist and bacterial contaminants.

Embryonic and larval development

Zygotes could only be obtained from egg masses that had been collected directly after oviposition. At this time,

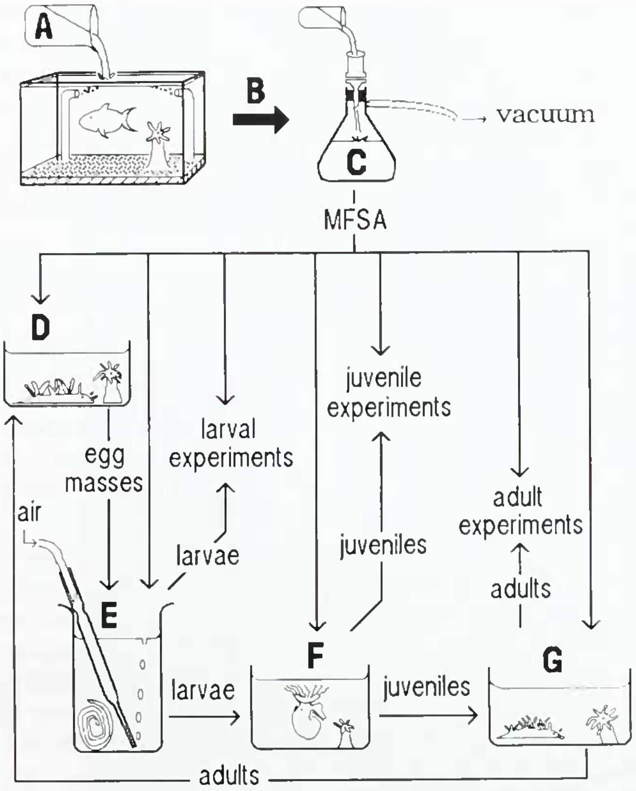

Figure 1. Algorithm for the culture of *Berghia verrucicornis.* (A) Aquaria with undergravel filtration and a 3–4 inch thick bed of $CaCO_3$ gravel are filled with freshly prepared artificial seawater. A few invertebrates or fish are added to each aquarium after 3–7 days of operation. After one month of operation, additional anemones or other animals are added to the aquaria. Salinity is adjusted each week. Water from these aquaria is designated as "seasoned aquarium water." (B) Seasoned aquarium water is regularly removed from established aquaria and used for egg mass, larval, juvenile and adult cultures. (C) Seasoned aquarium water is filtered through a 0.45-μm Millipore filter for culture use. (MFSA: Millipore-filtered seasoned aquarium water.) (D) Adults for stock cultures are maintained in small bowls (about 13 cm diameter, 5 cm deep). Bowl and water are changed daily. Appropriately sized anemone prey are added as needed. (E) Egg masses are collected from adult cultures and placed in 500-ml beakers containing ~350 ml of MFSA. Aeration is provided through Pasteur pipettes. Beaker and water are changed daily. As egg masses hatch, larvae are concentrated with a Nitex filter (see Switzer-Dunlap and Hadfield, 1981, p. 208) and used in larval experiments or metamorphosis cultures. (F) For metamorphosis cultures, larvae are placed in crystallizing dishes containing MFSA and a few small anemones. Water is left unchanged for 5–10 days and then the water is changed daily, and the bowl every few days as needed, until juveniles are large enough to transfer to finger bowls (2–3 weeks after metamorphosis). Juveniles are harvested for experiments or stock juvenile cultures. (G) Juvenile stocks are cultured as are the adults (see D above). Appropriate sized juveniles or adults are used for experiments or stock adult cultures. Transfer of larger juveniles and adults between cultures is easily accomplished with a "reversed" Pasteur pipette, the rubber bulb being placed over the end from which the tapered tip has been broken off.

the egg could be observed with a dissecting microscope, and its diameter measured with an ocular micrometer. Ten egg masses were cultured as described above and were observed at hourly intervals with a compound microscope until the cleavage divisions were finally obscured by the growing number of cells. Subsequently, until hatching, observations were made several times a day so that the appearance of characteristic larval structures, such as the velum, foot, left and right digestive diverticula, larval retractor muscles, eyespots, and propodium could be recorded. The number of egg masses laid per week was recorded for more than a month, for 22 adult nudibranchs.

Induction of metamorphosis

We assessed the effect of the sea anemone *Aiptasia pallida* on metamorphosis, by observing the following four metamorphosis cultures each containing 50 larvae: (1) MFSA in dishes containing the anemone *A. pallida* (MFSA + A); (2) MFSA in dishes without *A. pallida* (MFSA − A); (3) a bacterial film culture (BF) (see below); and (4) MFSA from an aquarium that had never contained *A. pallida* (*Aiptasia*-free, seasoned, aquarium water, AFSA). MFSA for treatments 1, 2, and 3 was taken from aquaria that contained *A. pallida.* Twenty replicates were performed for treatments 1 and 2, and ten replicates for treatments 3 and 4. New glassware was used to filter water and hold AFSA cultures. The bacterial film culture was prepared by removing a few pieces of gravel from an aquarium containing *A. pallida* and placing them in MFSA overnight. The next day the gravel was removed, the MFSA in the dish was changed and, finally, the larvae added. The number of larvae undergoing metamorphosis in each of the cultures was counted daily over three days. Care was taken to count all juveniles on the bottom, sides, and water surfaces of the culture. The mean ± standard deviation was calculated for each treatment and day. A Student *t*-test was used to determine whether the difference between the means was significant (Steel and Torrie, 1980).

Culture of egg masses for direct development

Single intact egg masses were cultured in dishes of MFSA without aeration. MFSA was changed daily in these cultures; the medium was decanted and fresh MFSA was gently added.

Results

Collection of adult animals

Berghia verrucicornis never inhabits the mangrove roots where its juvenile and adult food, the sea anemone *Aiptasia pallida,* occurs in abundance. Rather, the nudibranch is found in another habitat of *A. pallida,* among coral rubble in shallow, sub-tidal waters. The nudibranchs are relatively difficult to spot on the darkly colored coral rocks because the dorsally positioned cerata appear brown when the animal has been feeding. A typical adult of *B.*

verrucicornis is shown in Figure 2A. The white appearance of its egg mass (Fig. 2B) facilitates the collection of *B. verrucicornis* by providing evidence of the adults' presence.

The egg mass

The gelatinous egg masses are laid as untwisted strings in a dextral or sinistral spiral (Fig. 2B). In the laboratory, each pair of adults (n = 11 pairs) laid an average of 4 ± 1 egg masses weekly. The egg masses were found attached to the sides and bottom of the culture dish, and some were floating at the air-water interface; the site of oviposition seemed unaffected by the presence or absence of *Aiptasia pallida*. In the field, these egg masses are deposited on the underside of coral "rocks."

The embryos, whether in the field or in culture, are contained within two membranes: one, a primary membrane or capsule, surrounds each individual embryo; the secondary membrane encases all of the capsules (Fig. 2B, C). Empty primary egg capsules are located at both ends of the egg string.

Embryogenesis

Egg masses were cultured at 23.9 ± 1.3°C; the range was 21–26°C (Table I). Cleavage proceeded quickly at this temperature, and because the divisions within a given egg mass were asynchronous, both two-celled embryos and zygotes could be seen. This asynchrony was evident throughout cleavage, until the later blastula stage, when the opacity of the blastomeres reduced the accuracy of the cell count. All of the larvae hatched from the egg mass at the same morphological stage of development.

At 2.2 ± 0.3 days after oviposition, the velar rudiment was evident at the future anterior end of the embryo, and the embryo began to move. At first, the cilia were difficult to detect, and we could not be sure that they were beating. Nevertheless, the cilia are probably the cause of the movement of the embryos.

Larval structures

The velum is the first larval structure evident. It develops as a ridge on the anterior end of the embryo and assumes its characteristic bilobed appearance on the third day. The velar lobes are located anterolateral to the mouth and possess pre-oral and post-oral ciliary bands. By 4.7 ± 0.9 days, the embryo can partially retract the velum into the shell, indicating the presence of a retractor muscle. Large refractile cells, located around the periphery of the velum, are visible from day 8 until metamorphosis. The velum is lost during metamorphosis.

The larval foot (metapodium) appears soon after the velum, during the second day of development (2.8 ± 0.2 days), as a flat, blade-like metapodium. As development continues, the foot thickens and lengthens. The operculum is present by the fourth day. Cilia appear along the ventral length of the foot on the fifth day. The metapodium thickens considerably by the seventh day, and the posterior aspect develops into a definitive propodium soon thereafter (7.4 ± 0.5 days). During metamorphosis the foot becomes longer and wider; eventually it occupies the ventral surface of the juvenile.

The larval shell appears concurrently with the foot. The shell is clear, allowing an unobstructed view of the larval viscera. Shell length increases from 143.7 ± 21.4 μm on day 3 to a plateau of 251.4 ± 7.0 μm on day 8 (Fig. 3). When shell deposition has been completed, the mantle fold begins to withdraw from the shell edge. This occurs 7.0 ± 0.3 days after oviposition. The shell stopped growing once the mantle fold retracted, and was shed by the larva during metamorphosis.

The larval gut of *Berghia verrucicornis* occupies most of the space within the shell in the early days of development. It appears milky white and presumably contains yolk reserves that maintain the nudibranch throughout its embryonic and larval periods, and probably during early juvenile development. The various components of the viscera were not discernible early in development, but became evident 4.1 ± 0.2 days after oviposition. The stomach and the left and right digestive diverticula constituted most of the viscera. Initially, those three visceral organs are of similar size. As development proceeds, the right digestive diverticulum decreases in size (from 93.7 ± 9.6 μm on day 4, to 31.6 ± 5.0 μm on day 10), presumably reflecting the use of yolk reserves in this structure. The size of the left digestive diverticulum remains unchanged throughout development; Figure 4 shows the relationship between the diverticula from day 4 to day 10. The intestine could be seen looping anteriorly, from the posterior portion of the stomach to the anus on the right side of the mantle cavity, at the same time that the stomach and digestive diverticula were evident.

Sense organs present in the embryo and larva are the eyespots and statocysts. The statocysts are situated in the larval foot near its attachment to the body; they become apparent four days after oviposition. The two eyespots are located dorsally, directly posterior to the velar lobes; they appeared 6.3 ± 0.4 days after oviposition.

Hatching

Hatching occurred 11.6 ± 0.5 days after oviposition; the range was 9–14 days (T = 23.9 ± 1.3°C). In aerated laboratory cultures, the secondary egg mass membrane begins to break down first, releasing intact primary egg capsules. These primary egg capsules appear more pliable than earlier in development and change shape as the embryo moved within them. The primary egg capsules soon

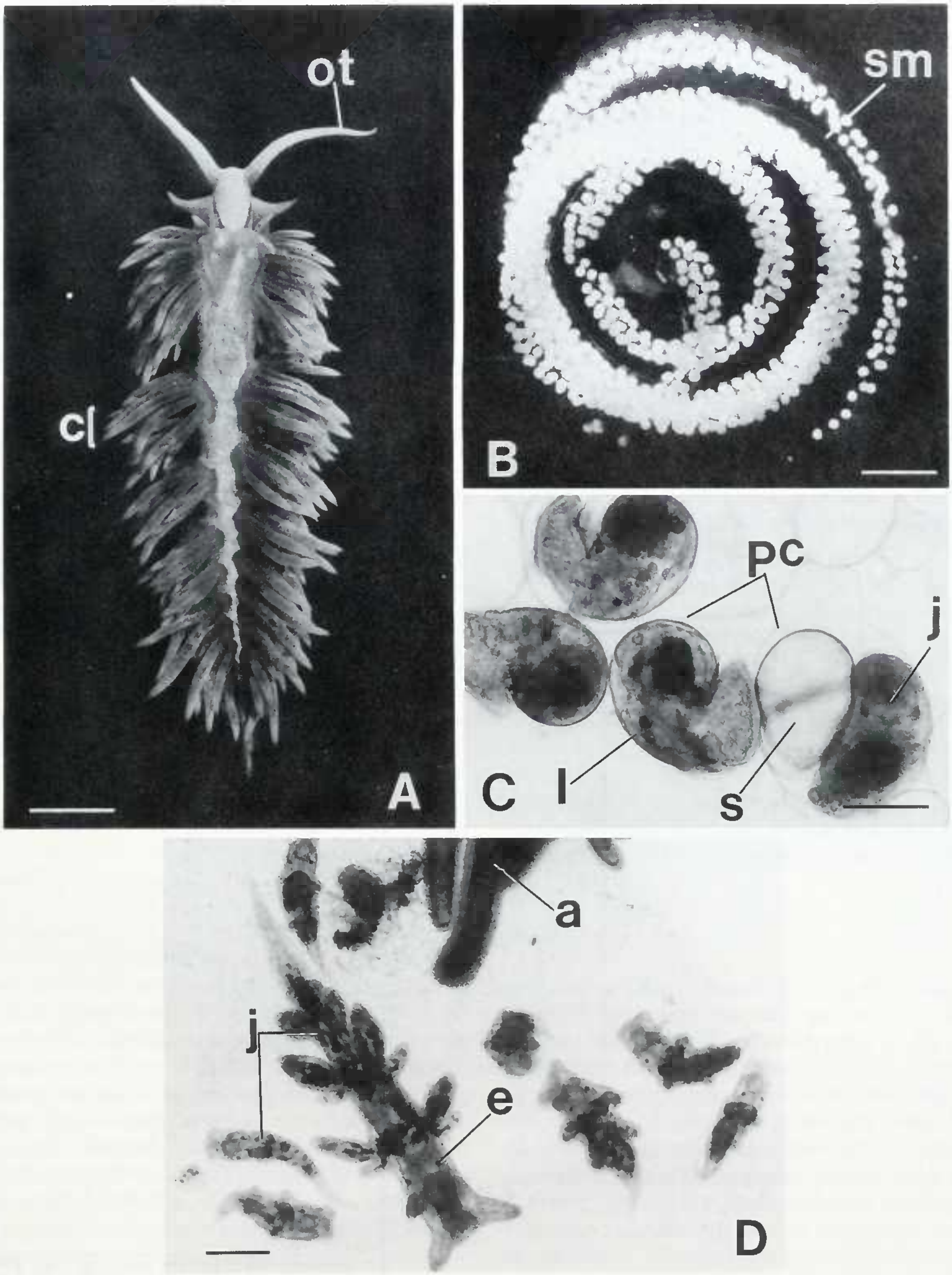

Figure 2. Photographs of several stages in the life history of *Berghia verrucicornis*. (A) Adult specimen. Scale bar = 2.0 mm. (B) Egg mass. Scale bar = 2 mm. (C) Example of direct development. A newly metamorphosed juvenile is still present in an egg capsule. Scale bar = 140 μm. (D) Juveniles of *Berghia verrucicornis*. Scale bar = 0.3 mm. Legend: a = anemone; c = ceratal tuft; e = eyespot; j = juvenile; l = larva; ot = oral tentacle; pc = primary capsule membrane; sm = secondary membrane; s = larval shell.

Table I

Developmental events during the laboratory culture of Berghia verrucicornis

Time* (h. days)		Developmental event
T = 23.9 ± 1.3°C		
0 h	(n** = 10)	Oviposition
3.4 ± 0.8 h	(n = 10)	First cleavage
14.5 ± 4.1 h	(n = 10)	Blastula
1.3 ± 0.2 days	(n = 10)	Gastrula
2.2 ± 0.3 days	(n = 10)	Velar rudiment appears
2.8 ± 0.2 days	(n = 10)	Shell and foot visible
3.3 ± 0.2 days	(n = 9)	Velum assumes bilobed shape
4.1 ± 0.2 days	(n = 9)	Viscera differentiated into stomach, and left and right digestive diverticula
4.7 ± 0.9 days	(n = 7)	Larva able to partially retract velum into shell, indicating presence of larval retractor muscle
5.5 days	(n = 2)	Cilia apparent on posterior aspect of metapodium
6.3 ± 0.4 days	(n = 9)	Eyespots visible
7.0 ± 0.3 days	(n = 9)	Mantle retracts from shell aperture
7.4 ± 0.5 days	(n = 9)	Propodium develops on anteroventral aspect of metapodium
11.6 ± 0.5 days	(n = 8)	Hatching
13.0 ± 0.9 days	(n = 8)	Metamorphosis
15.3 ± 0.8 days	(n = 6)	Juvenile begins feeding

* Times are given as mean ± standard deviation.

** n refers to the number of egg mass cultures observed; four cultures were discontinued during the observation period.

rupture, releasing veliger larvae. Neither the mouth nor the foot appear to participate actively in the hatching process.

Metamorphosis

The lecithotrophic larval stage of *Berghia verrucicornis* is released from the egg mass and undergoes metamorphosis as early as 1 day thereafter. Within metamorphosis cultures, the larvae swim vertically upward immediately after release from the primary egg capsules, and occasionally become trapped at the air-water interface. The larvae trapped at the water surface can move around because the velar cilia are submerged. They are apparently able to undergo metamorphosis without the benefit of attachment of the foot to the substratum. In metamorphosis cultures containing the anemone *A. pallida*, the larvae show no preference to settle near the anemones.

Marked changes in morphology take place during metamorphosis. After a short planktonic larval phase, generally 1–3 days, the larvae settle on the bottom and sides of the metamorphosis dish. Once settled, they appear to crawl randomly along the bottom, slowly beating their velar cilia. The velum is lost first, and the larvae continue to crawl on the substratum with the shell still attached. Eventually, the shell is cast off, and over the next few hours the body lengthens as the final vermiform morphology is assumed.

Various culture treatments were tested as inducers of metamorphosis (Fig. 5). The number of larvae completing metamorphosis was always greatest in the presence of the anemone *Aiptasia pallida* (MFSA + A culture). The mean cumulative number of larvae metamorphosing in MFSA + A cultures was significantly greater than the number of larvae undergoing metamorphosis in MFSA − A cultures ($P < 0.001$ for Day 1; $P < 0.01$ for Day 2 and 3) or in the AFSA cultures ($P < 0.01$ for all three days). However, cultures containing anemones (MFSA + A) did not differ significantly from the bacterial film (BF) cultures over the three day observation period ($P > 0.05$).

A comparison of the number of metamorphoses in the control cultures (BF, AFSA, and MFSA − A), revealed that the BF and AFSA cultures were significantly different only on Day 1 after hatching ($P < 0.05$). No difference was seen between the BF cultures and the MFSA − A cultures on any day tested ($P > 0.05$). Similarly, the number of larvae metamorphosing in the AFSA cultures was, statistically, the same as in cultures containing MFSA − A ($P > 0.05$).

Direct development

The hatching times for veliger larvae in aerated and unaerated cultures were not different ($P > 0.5$). The av-

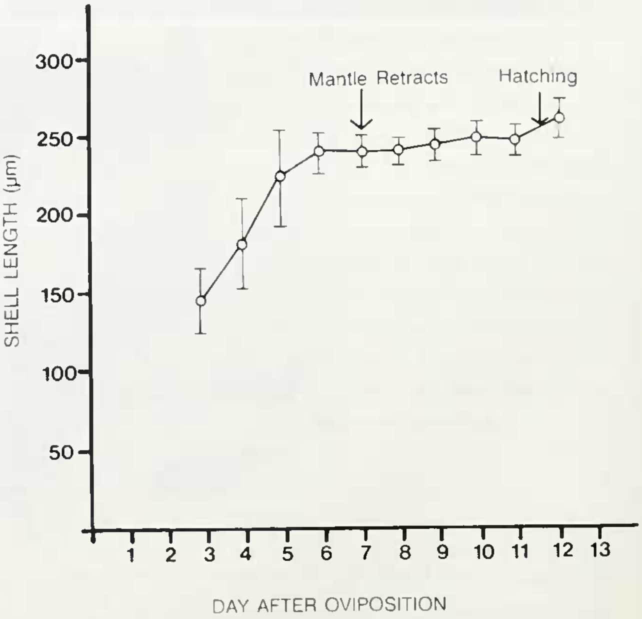

Figure 3. Changes in shell length during embryonic and larval development. n = the number of shell lengths measured.

Day =	3	4	5	6	7	8	9	10	11	12
n =	26	80	95	100	91	96	81	50	30	10

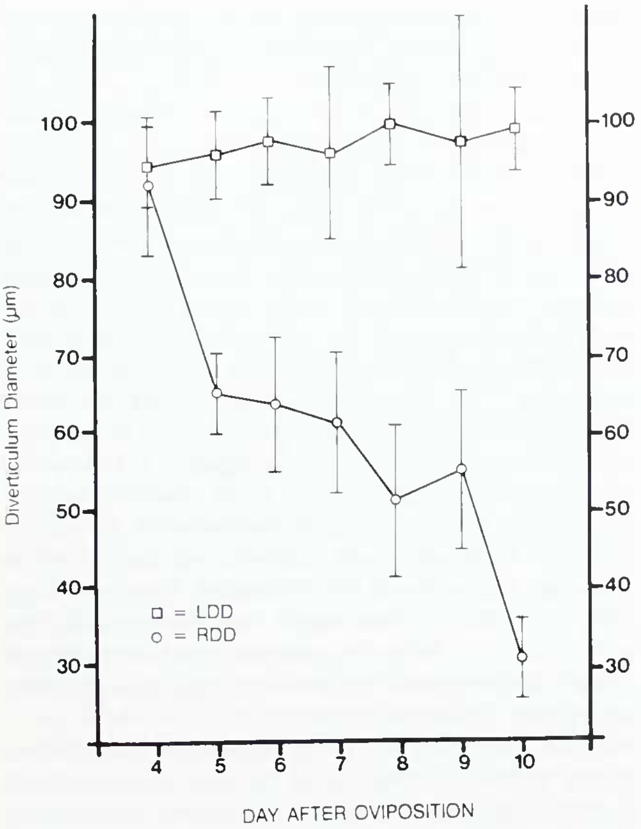

Figure 4. Changes in the diameter of the left and right digestive diverticula during embryogenesis. LDD, left digestive diverticulum; RDD, right digestive diverticulum. n = the number of diverticula measured.

Day =	4	5	6	7	8	9	10
n_{LDD} =	10	40	60	61	60	60	40
n_{RDD} =	10	20	30	31	30	18	5

erage time from oviposition to hatching in unaerated cultures was 11.7 ± 1.1 days (n = 30); in aerated cultures it was 11.6 ± 0.5 days (n = 8). However, both lecithotrophic larval and direct development were seen in egg masses from unaerated cultures (Fig. 2C). This type of variable development is known as poecilogony, which has been defined as "intraspecific variation in the mode of larval development" (Bouchet, 1989). In some instances, metamorphosis occurred within the primary egg capsule and the individuals hatched as juveniles, leaving the shell in the egg capsule; other capsules released lecithotrophic larvae that could metamorphose soon thereafter. Intracapsular metamorphosis occurred after some of the larvae had already hatched from the same egg mass. Veliger larvae hatching from the same egg masses underwent metamorphosis after a short planktonic period. The total number of hatchlings of each type varied considerably from one egg mass to the next. This may be due to differences between the embryos themselves, as the culture conditions were identical for each egg mass. The external appearance of juveniles was identical, whether development was direct or via a lecithotrophic larva.

Juvenile and adult

Newly metamorphosed juveniles were oval and white, with eyespots indicating the anterior end, and the remnants of the site of larval attachment to the shell at the posterior end. The juveniles initially crawled randomly over the bottom of the culture dish without feeding. One day after metamorphosis, a slender tail-like extension of the foot projected posterior to the elongated body of the juvenile. Rhinophore rudiments were present as small dorsal projections anterior to the eyes at this time. Stiff, cirrus-like projections extended from the anterior end of the juvenile and in opposed pairs along the dorsum. The foot was tightly associated with the body along the entire ventral length of the juvenile. In culture dishes, the juveniles gathered at the base of the anemones one day after metamorphosis. They then dispersed before returning one to two days later to begin feeding (15.3 ± 0.8 days after

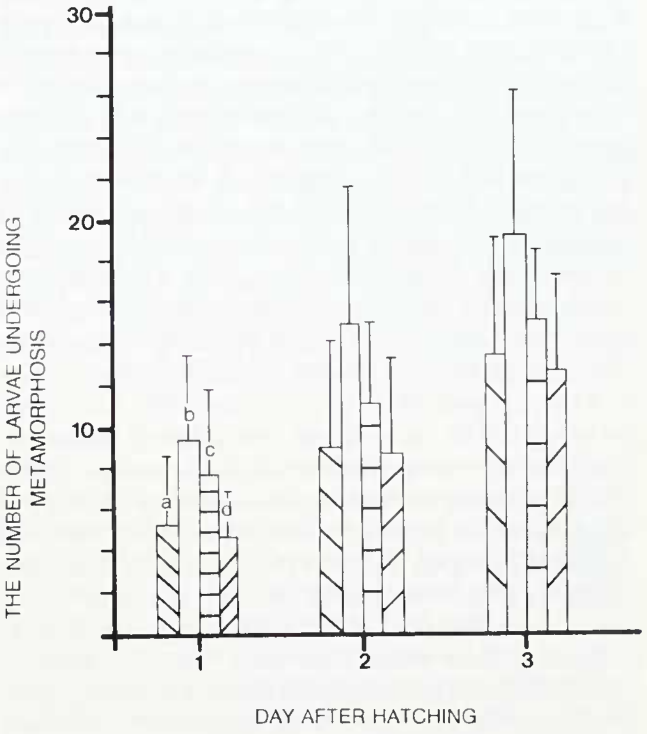

Figure 5. The mean cumulative number of larvae undergoing metamorphosis in four different culture media. Each culture contained 50 larvae initially. a = MFSA from aquaria containing *Aiptasia pallida*, but no anemones present (MFSA − A); b = MFSA plus the sea anemone *A. pallida* (MFSA + A); c = bacterial film culture (BF); d = *Aiptasia*-free, seasoned, aquarium water (AFSA). Twenty replicates were performed for treatments a and b, and 10 for treatments c and d. Error bars represent one S.D.

oviposition). The paired, dorsal cerata appeared 8–11 days after metamorphosis (Fig. 2D). Reproductive maturity was reached 50 ± 3 days after oviposition, but had been observed in the laboratory as early as 47 days. The first egg masses were small and contained less than 100 embryos.

Discussion

Berghia verrucicornis is the first opisthobranch mollusk to be cultured through successive generations and maintained as a viable population of experimental animals at an inland facility. This species has several characteristics that suggest it will be a useful model for laboratory oriented research. These include ease of maintenance, regular oviposition throughout the year (3 + egg masses/pair of animals/week), a prey organism (*Aiptasia pallida*) that can be cultured in the lab, a short embryonic (9–14 days) and lecithotrophic larval period (1–3 days), and a generation time (egg to egg) as short as 47 days. These traits should make *B. verrucicornis* a convenient organism for research in larval ecology, energetics, neurodevelopment, and, probably, neurophysiology. In addition, this species appears to maintain a symbiosis with a zooxanthella that it obtains from its prey (Kempf, 1989b), so the association can be used to investigate the establishment, energetics, and evolution of algal-invertebrate endosymbioses.

In general, the development of *Berghia verrucicornis* follows that reported for other opisthobranch mollusks with lecithotrophic larvae (Thompson, 1958, 1962; Bonar and Hadfield, 1974; Harris, 1975; and others). It is characterized by the major life cycle stages that Kriegstein (1977b) noted for *Aplysia californica*, *i.e.*, (1) embryonic, (2) planktonic, (3) metamorphic, (4) juvenile, and (5) adult. The morphological descriptions given for post-hatching stages of *A. californica* cannot be applied directly to *B. verrucicornis* because the larvae of *B. verrucicornis* are lecithotrophic and undergo what might be considered homologous developmental stages as an embryo, rather than as a feeding larva. In lecithotrophic larvae, such as these, the major increase in size of the viscera, development of the eyespots and propodium, and maximum shell length are all attained prior to hatching. In opisthobranch species with obligate planktotrophic larvae, such as *A. californica*, these events occur after hatching. Thus, *B. verrucicornis* might be included in the non-feeding, non-growing group of veliger larvae proposed by Hadfield (1963). But more recent investigations into the feeding potential of lecithotrophic larvae suggest that such distinctions may be hazy at best, because the lecithotrophic larvae of at least some species are capable of feeding if the opportunity presents itself (Kempf and Hadfield, 1985; Emlet, 1986; Kempf and Todd, 1989). A safer developmental designation would be that of type 2 larval development proposed by Thompson (1967); these larvae hatch from their egg capsules as late veligers, and undergo metamorphosis shortly thereafter.

The early cleavages of the aeolid nudibranch *Phestilla melanobranchia* are synchronized (Harris, 1975). Conversely, early cleavages in egg masses of *B. verrucicornis* are asynchronous. For instance, in a given egg mass, both zygotes and two-celled embryos can be observed simultaneously, although embryos more than one cleavage stage apart are never observed. Since this asynchrony in developmental stage is seen in embryos situated side by side, it is probably not due to differences in oxygen diffusion through the egg mass (Chaffee and Strathmann, 1984; Strathmann and Chaffee, 1984). Mediation of early development by a cue intrinsic to the egg mass (Harris, 1975) is a possibility, though the early development is certainly not as well synchronized as that reported for *P. melanobranchia*. As development proceeds, the discrepancy in cleavage rates becomes less noticeable, with later stages of *B. verrucicornis* developing at the same apparent rate. As a result, all sibling embryos incubated in an aerated culture hatch together at essentially the same morphological stage of development.

Lecithotrophic development, such as that characterized by the embryos and larvae of *Berghia verrucicornis*, is generally thought to use maternally derived yolk reserves to fulfill energetic needs. Recent investigations (Jaeckle and Manahan, 1989; Manahan, 1989; Manahan *et al.*, 1989; Shilling and Manahan, 1990) indicate that cellular endocytic systems in tissues other than those of the digestive tract may allow dissolved organic material from surrounding seawater to make a significant contribution to the energetic requirements of larval and possibly embryonic development. Although no conclusions with respect to the developmental importance of DOM can be drawn from this study, the decrease in size of the right digestive diverticulum during embryonic development in *B. verrucicornis* suggests that yolk reserves stored in this organ are an important nutrient source used to support embryonic energetic needs.

The mechanism of hatching has been described for several opisthobranchs. The aeolid, *Phestilla melanobranchia*, hatches through a hole made in the capsule wall by repeated contact with the mouth (Harris, 1975). In *Adalaria proxima*, a dorid, hatching appears to result from mechanical buffeting of the primary capsule wall with the velar cilia and, perhaps, the secretion of an enzyme, because the capsule wall becomes more pliable as the hatching date approaches (Thompson, 1958). Larvae of *Berghia verrucicornis* appear to effect their release from the primary egg capsule in a manner similar to that described for *A. proxima*. As hatching approaches the capsule membrane becomes more flexible. Continuous buffeting and distortion of this barrier by the velar cilia may be sufficient to eventually tear an opening that allows release of the larva. Bacteria, protozoans, and other microfauna may also contribute to the lysis of egg mass membranes during hatching.

In opisthobranchs, as in many other invertebrates, external cues are often responsible for the onset of metamorphosis (Thorson, 1946; Thompson, 1958, 1962; Bonar and Hadfield, 1974; Harris, 1975). Metamorphosis of the larvae of such species is triggered by chemicals or factors associated with a specific aspect of the mature animals habitat, often the presence of the food of adults (Hadfield, 1977, 1984, 1986; Burke, 1983, 1986; Hadfield and Scheuer, 1985; Morse, 1985; Fitt *et al.*, 1987; and many others). Our experiments demonstrate that a habitat-specific inducing factor is not an absolute requirement for *Berghia verrucicornis*. A mean of 26% of the veligers released from the egg masses of this species metamorphosed in the absence of a habitat-specific inducing molecule (AFSA cultures). But the treatments used to examine induction do not preclude the presence of some ubiquitous inducing molecule common to seawater or to habitats in general (*e.g.*, from bacterial or algal films). Further analysis of our experiments on metamorphic induction requires consideration of three components of the results. (1) The presence of the anemone *Aiptasia pallida* always resulted in the greatest number of larvae completing metamorphosis. (2) The number of larvae metamorphosing in anemone-containing treatments was always significantly different from that in controls containing only (a) Millipore-filtered, seasoned aquarium water from aquaria containing *A. pallida* (MFSA − A), or (b) *Aiptasia*-free, seasoned aquarium water (AFSA). (3) Bacterial film controls were only different from the AFSA controls on the first day after hatching. These results suggest that a factor (presumably chemical) enhancing a larva's tendency to metamorphose is associated with the anemone *A. pallida*. The enhancement of metamorphosis by a substance associated with the prey of juveniles and adults, rather than a habitat-specific metamorphic induction, would be consistent with proposals recently made by Kempf and Todd (1989) concerning the functional aspects of evolutionary selection for direct development (see below).

The juveniles that gather at the base of the anemones one day after metamorphosis may be responding to the same anemone-associated factor that enhances metamorphosis. The presence of such a response would help to ensure that they gathered in areas containing their prey organism. After the initial "discovery" of the anemone, juveniles in culture bowls disperse, presumably searching out other areas where prey are present. The re-aggregation of juveniles at the bases of culture bowl anemones 1–2 days after dispersal may well be an artifact produced by confinement in the culture container. The nudibranchs simply "rediscovered" the same anemones in the small container and began feeding as their maternally derived yolk reserves were depleted.

Classically, most invertebrate species have been considered to undergo a single type of development; but reports of poecilogony occurring in a number of invertebrate species, including opisthobranchs, suggest that intraspecific developmental flexibility is greater than was previously thought possible (Clark *et al.*, 1978; Eyster, 1979; Gibson and Chia, 1989; Hoagland and Robertson, 1988 review). Our observations indicate that, when egg masses of *Berghia verrucicornis* are agitated by aeration, only veliger larvae hatch from the primary egg capsules. An inadvertently forgotten culture in our lab led to the serendipitous observation that, if egg masses of this species are left undisturbed by aeration, both veligers and juveniles will be released from primary capsules of the same egg mass. Repeated observations of additional unaerated egg masses suggest that the factors affecting the type of development expressed by egg masses of *Berghia verrucicornis* may be extrinsic rather than intrinsic. One explanation for this phenomenon is that aeration of egg mass cultures causes a mechanical break down of egg mass membranes, resulting in the "premature" release of only veliger larvae from the primary egg capsules. Lack of aeration (*i.e.*, agitation) would allow for greater integrity of the egg mass membranes, thus allowing time for some embryos to undergo metamorphosis within the primary capsule. This scenario seems unlikely, because aerated and unaerated cultures show no significant difference in time to hatching at similar temperatures. Perhaps a more acceptable explanation is that agitation due to aeration somehow interferes with either (a) the physical act of metamorphosis itself or, (b) the intrinsic larval systems responsible for attaining competence or initiating metamorphosis. Whether the poecilogony described above actually occurs in the field has not been determined; but, two facts suggest that it does: (1) *Berghia verrucicornis* lays its egg masses closely attached, along the egg string's entire length, to the bottoms of coral rocks, thus reducing the effects of agitation; and (2) egg masses are laid in subtidal habitats of low energy flux in some instances. Thus, *Berghia verrucicornis* may enjoy the best of two worlds, being able to benefit from both the advantages of direct development and dispersal via a larval stage.

Kempf and Todd (1989) have proposed that, as a species evolves toward a reproductive strategy characterized by lecithotrophic development, a loss in habitat-specific induction of metamorphosis would usually be necessary prior to the establishment of a direct mode of development. *Berghia verrucicornis* appears to exhibit developmental characteristics that are consistent with this hypothesis. It has lost the need for a habitat-specific inducer and, given the right environmental conditions, can reproduce via both a larval stage and direct metamorphic development as described by Bonar (1976). As such, *B. verrucicornis* appears to be poised on the cusp between indirect (larval) and direct development, suggesting that it will be useful in future studies concerning the evolution of invertebrate reproductive strategies.

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Development of a True Ovoviviparous Sea Star, *Asterina pseudoexigua pacifica* Hayashi

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Abstract. *Asterina pseudoexigua pacifica* is a true ovoviviparous asteroid in that its development and metamorphosis occur within the spatial hermaphroditic gonad. From the middle of June to the middle of July, the gonad contains numerous embryos and juveniles in various stages through metamorphosis. The opaque, greenish yellow mature ovum is 450 μm in diameter. Development is direct. Embryos develop through wrinkled blastula and gastrula stages into a pear-shaped brachiolaria with three arms. The general process is similar to that of asteroids having direct development. Newly metamorphosed juveniles are released from the gonopores. Peak release occurs in the middle of July. The maximum number of juveniles released from an adult is about 1300. The juvenile is 900 μm in diameter and has two pairs of tube-feet in each arm; the skeletal plates are well developed. The present results are compared with those of other true viviparous echinoderms.

Introduction

The specific name *vivipara* has often been given to species that are believed to be viviparous, and a number of echinoderm species bear this name. Hendler (1979) noted about 60 viviparous ophiuroids, and many holothuroids also have this mode of development. The term "viviparity," however, should be restricted to the case in which embryos develop within the gonad or the genital tract, a portion of which may be specialized for incubating embryos. Coelomic or bursal incubation is a specialized type of brooding. If we accept this definition, only five species of echinoderms are known to be viviparous: Ophiuroidea, *Ophionotus hexactis* (by Mortensen, 1921), Holothuroidea, *Leptosynapta clarki* (by Everingham, 1961; cited from McEuen, 1987) and *Taeniogyrus contortus* (by Boolootian, 1966), Asteroidea, *Patiriella vivipara* (by Dartnall, 1969; Chia, 1976), and Concentricycloidea, *Xyloplax medusiformis* (by Rowe *et al.*, 1988). Descriptions of reproduction and development in these species, although sufficient to establish viviparity, are fragmentary.

Development through metamorphosis is known in about 40 of the 2500 extant asteroid species. Among these, reproduction and development are best known in several species of *Asterina*. The entire development through metamorphosis has been reported in *A. gibbosa* (Ludwig, 1882; MacBride, 1896), *A. coronata japonica* (Komatsu, 1975), *A. batheri* (Kano and Komatsu, 1978), and *A. minor* (Komatsu *et al.*, 1979). Larval development has been reported in *A. exigua*, *A. pectinifera*, and *A. regularis* (Mortensen, 1921; Komatsu, 1972). Sexuality and reproduction have been reported in some asterinid species (Cuénot, 1898; Ohshima, 1929; Bacci, 1949; Cognetti, 1954; Delavault, 1966; Dartnall, 1970; Emson and Crump, 1979; Komatsu *et al.*, 1979). Some species have direct development (yolky eggs and development only through the brachiolaria stage), while others have indirect development (non-yolky eggs and development through both the bipinnaria and brachiolaria stages). As to the sexuality of asterinids, some are gonochoric and some are hermaphroditic. Among the latter are *A. batheri*, *A. gibbosa*, *A. minor*, *A. pancerii*, *A. phylactica*, and *A. scobinata*. *Asterina minor* shows a breeding assemblage (Komatsu *et al.*, 1979). Thus, the diversity of development and reproduction occurring in various species belonging to the genus *Asterina* is well documented.

Asterina pseudoexigua pacifica was described by Hayashi (1977) as a new subspecies of *A. pseudoexigua* Dartnall. This subspecies differs from *A. pseudoexigua* in that its gonopores are on the abactinal side, and it is ovoviviparous. Development through metamorphosis takes

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Table I

Number and size distribution of juveniles of* Asterina pseudoxigua pacifica *collected in the field*

Number of tube-feet in the longest arm (in pairs)	Individual Number											
					April			May		June		
	July 30, 31 1974	Sept. 11, 12 1973	Dec. 7, 8 1972	Feb. 16 1976	23 1986	25 1975	28 1976	1, 2 1985	14, 15 1973	13 1976	21–25 1987	27–30 1986
2	37											
3	4	1		1		3	1				2	
4		9		5	6	6	3	2			13	1
5		9		20	8	7	11	14	4	2	29	7
6		4		34	16	8	26	45	1	6	58	22
7		1	2	16	15		28	56	2	11	32	34
8		1	8	4	5	1	11	30	1	19	36	21
9			12	2	1		1	10			32	9

* Juveniles bear fewer than nine pairs of tube-feet on the longest arm.

place in the gonad, and juveniles are released from the adult. The present report describes ovoviviparity and the entire process of development of *A. pseudoexigua pacifica.*

Materials and Methods

From time to time, between 1974 and 1987, specimens of *Asterina pseudoexigua pacifica* Hayashi were collected from the undersurface of stones in the intertidal zone of Kushimoto, Wakayama Prefecture (Fig. 1A). The number and size distribution of the juveniles collected are given in Table I. The specimens were kept alive in the laboratory.

Adults were cultured individually in small glass jars so that the release of embryos could be observed. During culture, the temperature was maintained similar to that of the natural habitat (20–25°C).

Embryos of various stages of development were obtained by dissecting the gonad at appropriate intervals between the middle of June to the middle of July.

General observations were made using dissecting and light microscopes. Living embryos were measured with an ocular micrometer. For microscopic examination of the skeletal system, juveniles were fixed in 70% alcohol, then macerated in a 10% aqueous solution of potassium hydroxide. For histological observations of the gonad, some specimens were fixed with Bouin's solution immediately after collection. The fixed material was embedded in paraffin, serially sectioned at 6 μm, and stained with Delafield's hematoxylin and eosin.

Gonads and embryos obtained from the gonad by dissection were fixed for scanning electron microscopy in 2% OsO_4 in 50 m*M* Na-cacodylate buffer (pH 7.4); the osmolarity of the fixative was adjusted by the addition of sucrose (final concentration, 0.6 *M*). The fixed materials were dehydrated in ethanol, dried with a critical-point dryer (Hitachi, HCP-2), and observed with a scanning electron microscope (Hitachi, S-510) after being coated with gold-palladium (Hitachi, E101 Ion Sputter).

Results

Ovoviviparity

The gonad is composed of clusters of lobules arranged in pairs in each interradius. Each gonad opens on the aboral side of the disk through a gonoduct (Fig. 1B). In early June, each gonad consists of ovarian and testicular portions (Fig. 1C, D). The majority of the eggs in the ovarian part are fully grown, nearly spherical (about 400 μm in diameter), and pale green. The head of the sperm contained in the testicular part is spherical (2–3 μm in diameter); the total length, including the tail, is 50 μm. This species is a spatial hermaphrodite, containing full-grown ova and active sperms simultaneously in each gonad.

The breeding season of *Asterina pseudoexigua pacifica* is from the middle of June to the middle of July, and most adults have many developing embryos or juveniles in their gonads. Developing embryos of particular stages can be obtained if the gonad is dissected at the appropriate time during the season: early cleavage and early gastrula stages in the middle of June (Fig. 2A, B); late gastrula stage from the middle to the end of June (Fig. 2C); brachiolariae from the end of June to the beginning of July (Figs. 1G, 2D and 2E); and metamorphosing larvae and juveniles from the beginning to the middle of July (Figs. 1H; 2F, G). There are some individual and yearly variations.

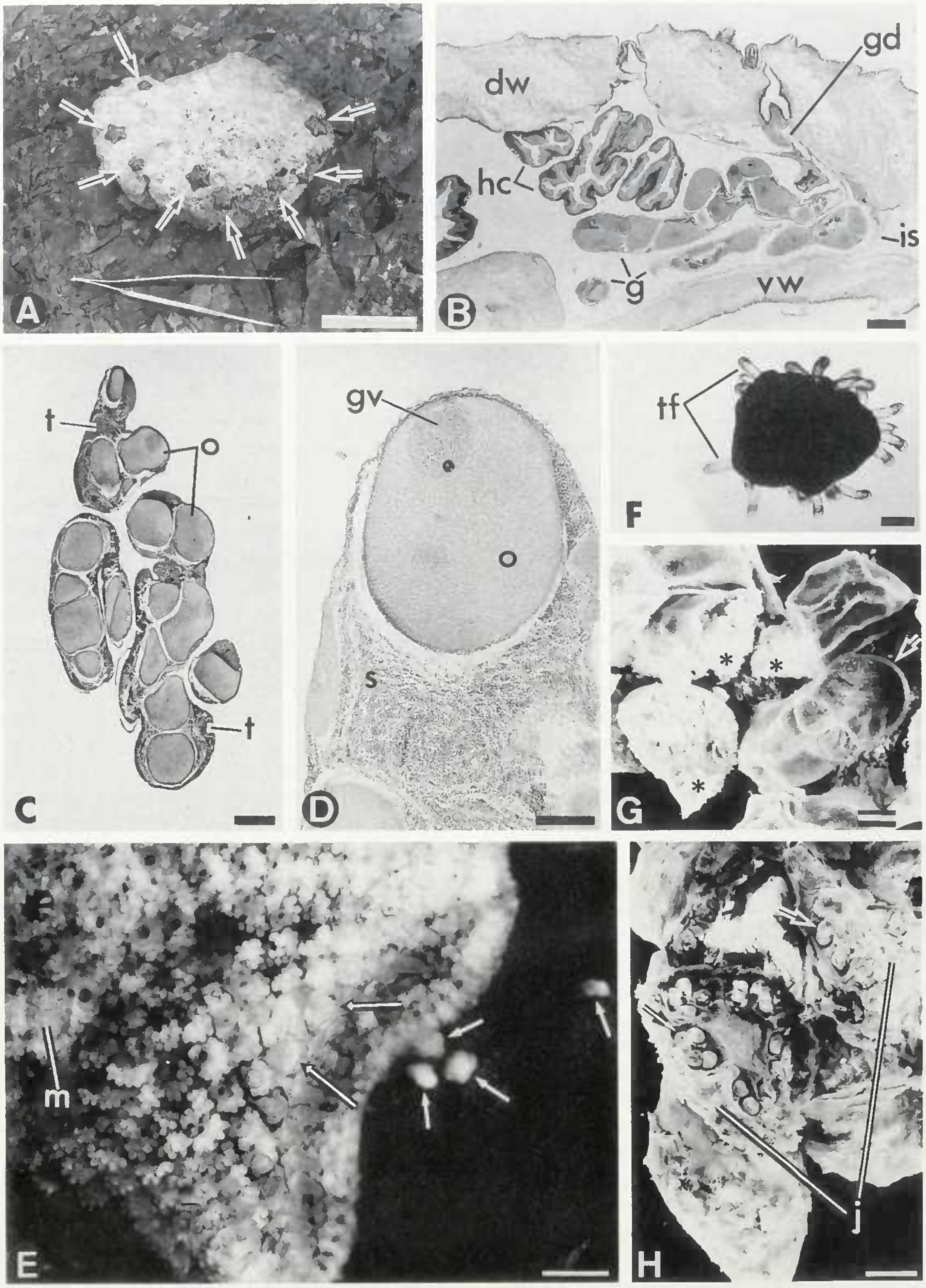

Figure 1. A. Specimens (arrows) of adult *Asterina pseudoexigua pacifica*, attached to the undersurface of a stone on the shore of Kushimoto. Bar scale = 50 mm. B. Sagittal section of the gonad (g) of *A. p. pacifica*. Note gonoduct (gd) passing through the dorsal wall (dw). Bar scale = 200 μm. hc, hepatic caecum; is, interradial septum; vw, ventral wall. C. Section of the hermaphroditic gonad of a specimen of *A. p. pacifica*. Note full grown ova and mature sperms. Bar scale = 200 μm. o, ovum; t, testicular portion. D. Magnified picture of the hermaphroditic gonad. Bar scale = 100 μm. gv, germinal vesicle; o, ovum; s, sperm.

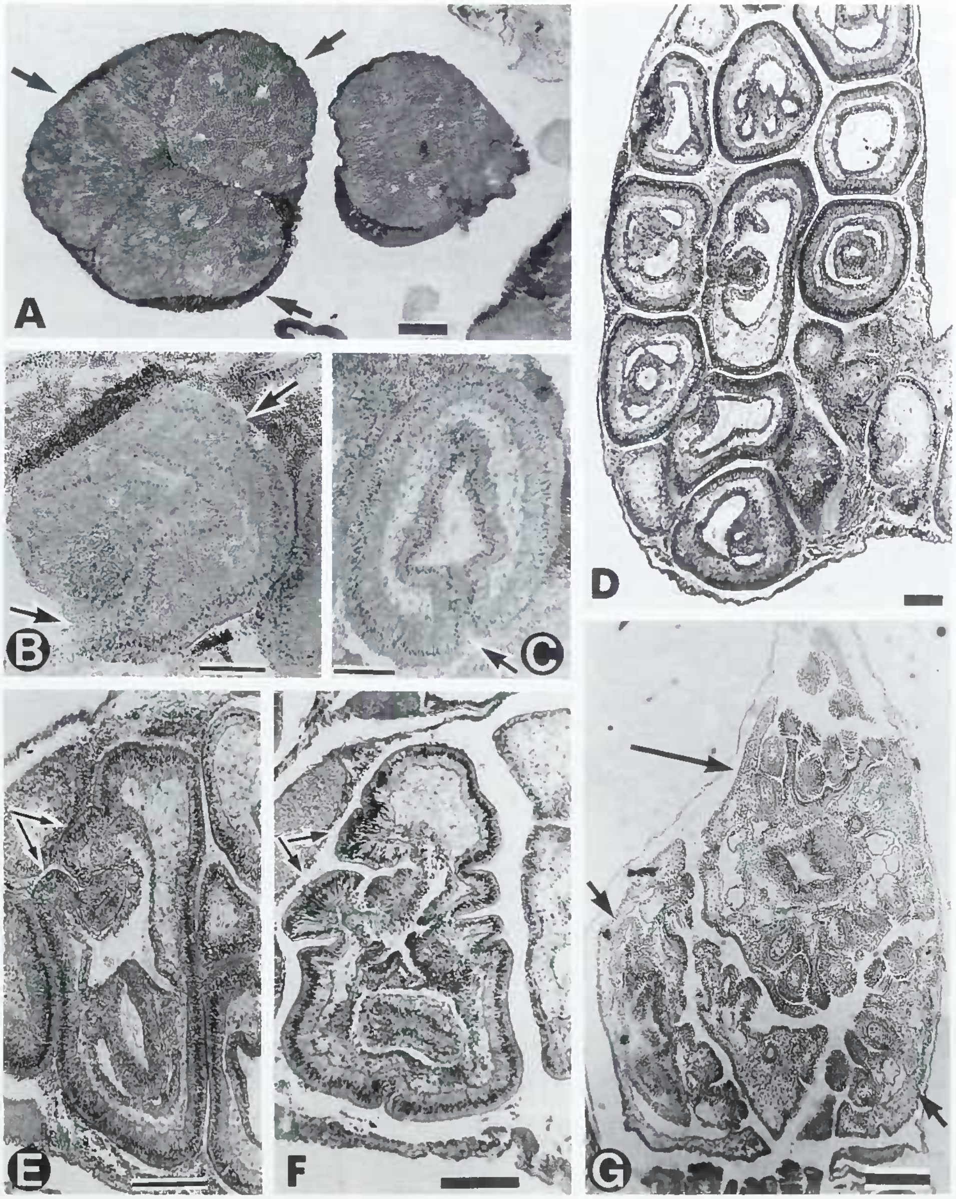

Figure 2. Micrographs of sections of the gonad of a specimen of *Asterina pseudoexigua pacifica*. Bar scale = 100 μm. A. Arrows point to three embryos in early cleavage. B. Wrinkled blastula with grooves (arrows). C. Sagittal section of a gastrula with a differentiated archenteron. Arrow indicates blastopore. D. Many embryos developing simultaneously in the gonad. E. Sagittal section of a brachiolaria with brachiolar arms (arrows). F. Sagittal section of a metamorphosing brachiolaria. Arrows show brachiolar arms. G. Horizontal (long arrow) and cross (short arrows) sections of juveniles in the gonad.

E. Dorsal view of a birthing specimen of *A. p. pacifica*. Short and long arrows indicate juveniles after birth and just appearing from gonopores, respectively. Bar scale = 1 mm. m, madreporite. F. Living specimen of a juvenile of *A. p. pacifica* just after birth, dorsal view. Bar scale = 200 μm. tf, tube-foot. G. Scanning electron micrograph of the inside of the gonad of a specimen of *A. p. pacifica*. Note brachiolaria (arrow) and metamorphosing larvae (asterisks). Bar scale = 100 μm. H. Same as Figure 1G, showing juveniles (j) with tube-feet (arrows) just prior to birth.

Development

Developing embryos of various stages are easily obtained by dissecting the gonads during mid-June to mid-July. The embryos usually fill the coelom of the adult. The developmental stages shown in Figures 3 and 4 represent embryos removed from dissected gonads; organogenesis (Fig. 2) was studied in sections of the gonad. Cleavage is total, equal, and radial (Fig. 4A). The early blastula is 450 μm in diameter and composed of equal-sized blastomeres (Fig. 4B). Figures 3C and 4C show blastulae in the most wrinkled stage. The surface of the blastula is divided by furrows into several portions, each consisting of clusters of blastomeres (Fig. 2B). Gastrulation takes place by invagination; the blastopore is circular and small (30 μm in diameter). Early gastrulae are 440 μm long and 380 μm wide. Hatching must follow the wrinkled blastula stage, because no fertilization membrane is ob-

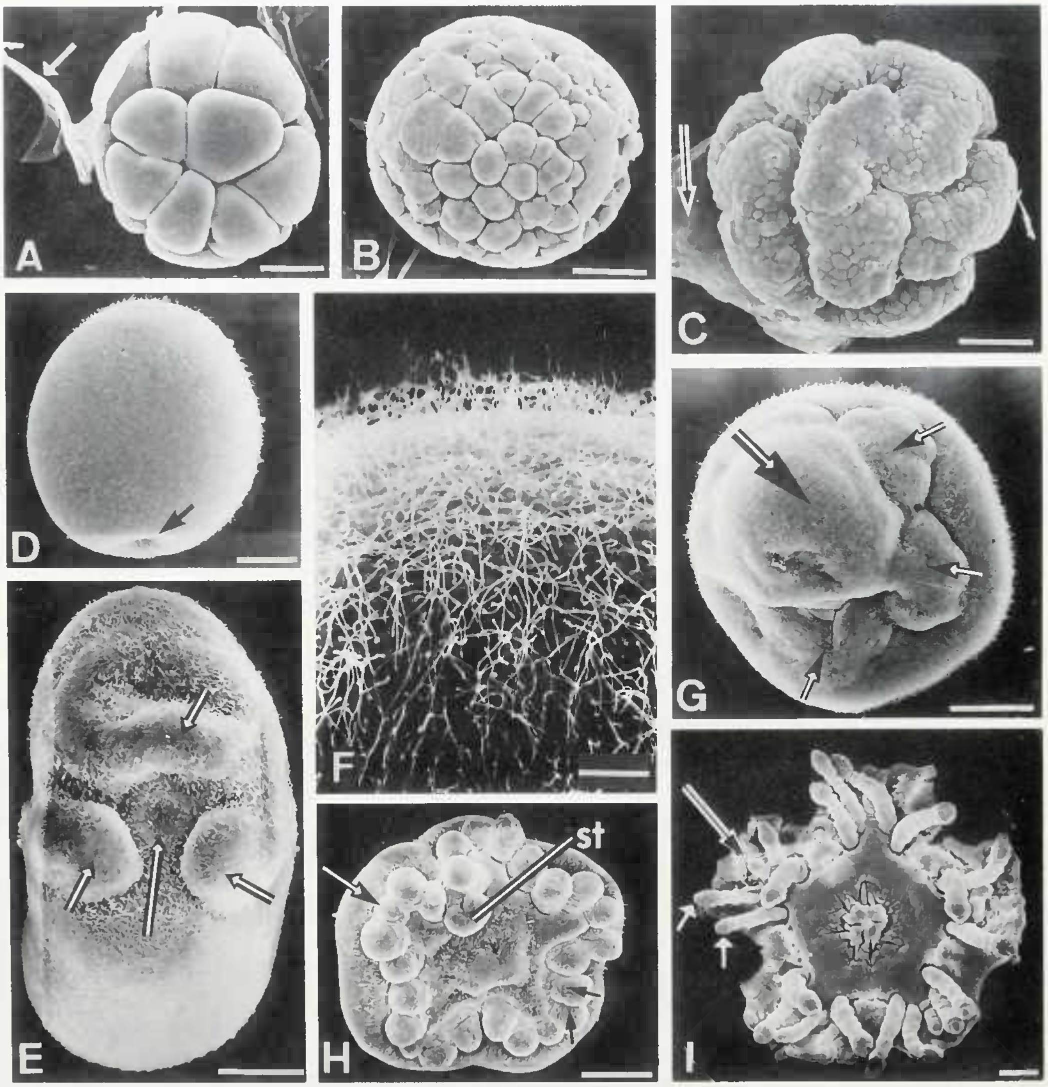

Figure 3. Scanning electron micrographs of specimens of *Asterina pseudoexigua pacifica.* The specimens shown in A–H were dissected out of the gonad. The juvenile shown in I was born from the gonopore. Bar scale = 100 μm. A. Embryo of an early cleavage with a fragment of the removed fertilization membrane (arrow). B. Early blastula. C. Wrinkled blastula in its most conspicuous stage. Arrow shows a fragment of the removed fertilization membrane. D. Early gastrula with blastopore (arrow) after hatching. E. Ventral side of a brachiolaria bearing three brachiolar arms (short arrows) and a central sucker (long arrow) among them. F. Magnified view of the anterior part of the specimen shown in Figure 3E, illustrating ciliation. G. Anterior view of the metamorphosing larva. Long and short arrows indicate stalk of larva and hydrolobes, respectively. Each hydrolobe has rudiments of a terminal tentacle and two pairs of tube-feet. H. More advanced metamorphosing larva with terminal tentacle (long arrow) and tube-feet (short arrows). st, stalk of larva. I. Juvenile after birth. Long and short arrows show a terminal tentacle and tube-feet, respectively.

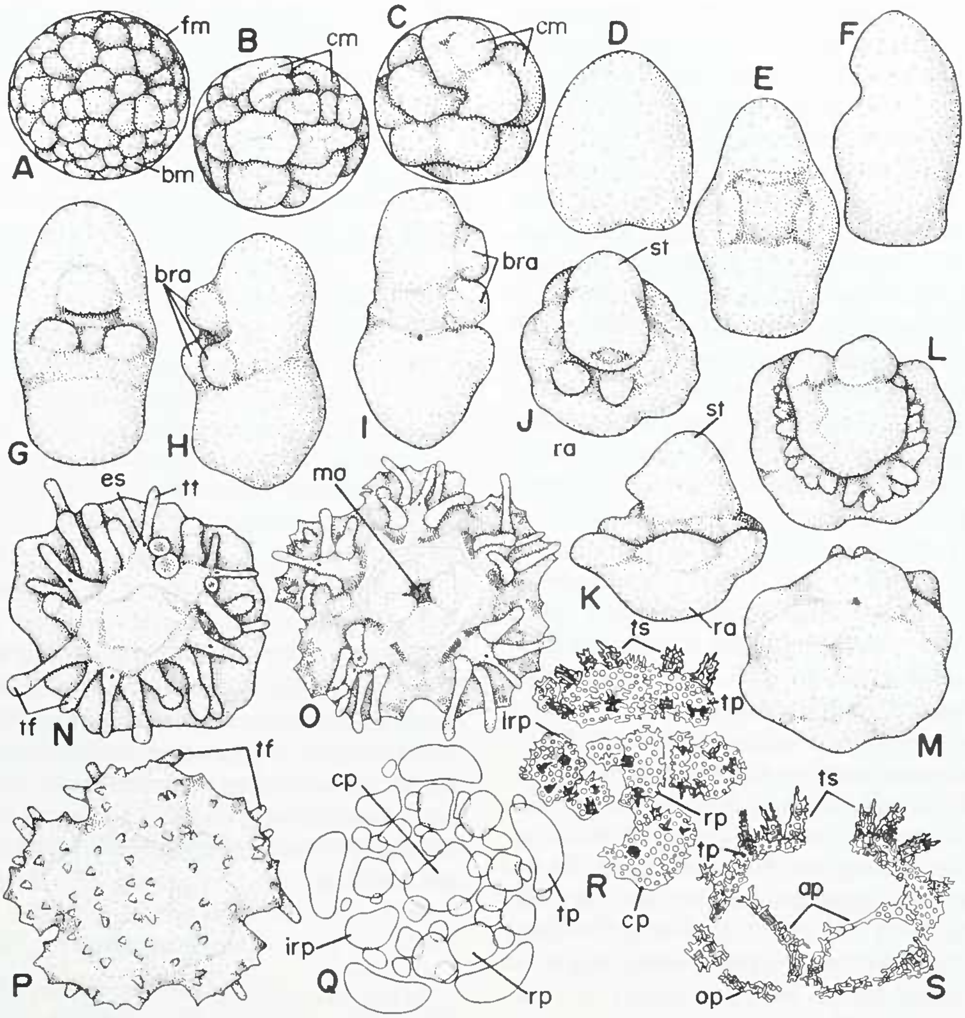

Figure 4. Development of *Asterina pseudoexigua pacifica*. Every drawing was made from a living specimen. Specimens in A–N and in O–S were either dissected out of the gonad, or born from the gonopores, respectively. A. Early blastula, earlier stage than that shown in Figure 3B. bm, blastomere; fm, fertilization membrane. B. Wrinkled blastula in earlier stage than that shown in Figure 3C. cm, cell mass. C. More advanced wrinkled blastula than that shown in Figure 3C. cm, cell mass. D. Early gastrula, same stage as shown in Figure 3D. E. Early brachiolaria, ventral view. F. Same as Figure 4E, left lateral view. G. Brachiolaria in earlier stage than that shown in Figures 3E and 4I. H. Same as Figure 4G, ventro-lateral (left) view. bra, brachiolar arm. I. More advanced brachiolaria, same stage as shown in Figure 3E, right lateral view. J. Metamorphosing larva in earlier stage than that shown in Figure 3G, anterior (future oral) view. st, stalk of larva. K. Same as Figure 4J, left lateral view. ra, rudiment of adult; st, stalk of larva. L. More advanced metamorphosing larva than that shown in Figure 3G, future oral view. M. Same as Figure 4L, future aboral view. N. Metamorphosing larva just before completion of metamorphosis, future oral view. es, eye-spot; tf, tube-foot; tt, terminal tentacle. O. Juvenile after birth, same stage as shown in Figure 3I, oral view. mo, mouth. P. Same as Figure 4O, aboral view. tf, tube-foot. Q. Schematic drawing of aboral skeletal system, same stage as shown Figure 4O. cp, central plate; irp, interradial plate; rp, radial plate; tp, terminal plate. R. Skeletal plates and spines of a ray of a specimen shown in Figure 4Q, aboral view. cp, central plate; irp, interradial plate; rp, radial plate; tp, terminal plate; ts, terminal spine. S. Same as Figure 4R, oral view. ap, ambulacral plate; op, oral plate; tp, terminal plate; ts, terminal spine.

served around the gastrula. Coelomic pouches emerge from the tip of the archenteron during the gastrula stage (Fig. 2C). Many mesenchyme cells are present in the blastocoel.

The larva of this sea star is a pear-shaped brachiolaria. Early brachiolariae with rudiments of brachiolar arms are shown in Figure 4E and F. Brachiolariae, which grow to become 600 μm long and 350 μm wide, bear three apparent brachiolar arms (Fig. 4G, H). Brachiolar arms are short; the lengths of the ventro-anterior arm and of the ventro-lateral arms are about 150 μm and 75 μm, respectively. At this stage, the blastopore is closed. Brachiolariae taken from the gonad can swim in seawater. The body surface of the larva is covered by cilia (Fig. 3E); they are about 10 μm long and are uniformly distributed at about 15/100 μm^2. Figures 2E, 3F, and 4I show more developed brachiolariae than those shown in Figure 4G and H. The posterior part of the larval body of this stage, which corresponds to the larval disk, becomes transformed into a subpentagonal form. A small hydropore is present near the center of the right side of the body. Three brachiolar arms become longer and project beyond the "central sucker," the triangular region defined by the bases of the three arms. The ventro-anterior arm is 175 μm long and ventro-lateral arms are 100 μm. The anterior part of the body, designated as the "stalk" of the larva, becomes translucent except for the tip of the brachiolar arms.

At metamorphosis, the stalk is absorbed (Fig. 2G). The posterior portion of the metamorphosing larva is hemispherical with a subpentagonal margin, being 450 μm in diameter. The metamorphosing larva, shown in Figure 4J and K, bears the shrunken stalk. The bulges of the hydrolobe become recognizable on the future oral side of the disk. Three brachiolar arms are still distinguishable at this stage. Tube-feet appear on the future oral side of the body in more advanced larvae (Fig. 4L, M). At this stage, the stalk is further reduced and situated in one interradius (Fig. 3H). The larva shown in Figure 4N has almost completed metamorphosis, and its diameter is 650 μm. The stalk has been completely absorbed. Two pairs of tube-feet and one terminal tentacle, which has a red eye-spot at the basal portion, are developed in each ray (Fig. 2G). When removed from the gonad, the larvae use their tube-feet to move on the substratum.

Release

Three adults, collected 4–7 July 1974 and kept individually in small jars, began to release juveniles from their gonopores on 10 July (individuals A, C, and I in Table II; Fig. 1F). Soon after release, the juveniles leave their mother and move around on the substratum with their tube-feet. The juveniles, about 900 μm in diameter, are white with a yellow tint (Figs. 1E; 4P, Q). They have two pairs of tube-feet in each arm, and their mouths are open. Skeletal plates (1 central, 5 interradial, and 5 radial plates on the aboral side; 5 pairs of oral and ambulacral plates on the oral side; and 5 terminal plates) are well developed (Fig. 4R, S, T). The release of juveniles by adults in the laboratory after mid-July has been observed since 1975. The number of juveniles released from adults is shown in Table II. The peak season of release is from 11 to 20 July. The maximum number of juveniles born from one adult was 1288.

Table II

Number of juveniles released from an adult of Asterina pseudoxigua pacifica

Individual	July 10	July 11–20	July 21–31	August 1–10	Total
A	20	1,179	82	7	1,288
B		996	4	15	1,015
C	3	957	1		961
D		263	3	32	298
E		257	1	2	260
F		158			158
G		113			113
H		63	17		80
I	3	50	16		69
J		46			46
K		28	5	9	42
L			17	22	39
M		35			35
N		6	9		15

Most juveniles collected at the end of July 1974 (Table I) had two pairs of tube-feet on each arm, so they had been born less than one month previously. In September and February, the juveniles collected from the field are on average larger than those collected in July. We conclude from these data that juveniles have more than five pairs of tube-feet (some with 6 or 7 pairs) in each arm one year after birth.

Discussion

Many echinoderms have been described as viviparous. These include 2 species of crinoids, 7 of holothuroids, and 70 of ophiuroids. *Isometra vivipara,* a crinoid, broods its eggs in a chamber, formed by pinnules, called a marsupium (Andersson, 1904; Mortensen, 1920). *Chiridota rotifera,* a holothuroid, broods its eggs in the coelom (Clark, 1910; Boolootian, 1966). *Stegophiura sculpta,* an ophiuroid, broods its larvae in the bursa (Murakami, 1941). But none of these species is truly viviparous, because development proceeds outside of the ovary or the genital tract.

Mortensen (1921) reported that development in *Ophionotus hexactis* begins in the ovary and, indeed, that the embryos develop entirely within the ovary. Everingham (1961; cited from McEuen, 1987) observed that *Leptosynapta clarki* is an intraovarian brooder. *Taeniogyrus contortus* was listed by Boolootian (1966) as a presumptive ovarian incubator. Dartnall (1969) reported that in *Patiriella vivipara* the embryonic development occurs in a sac derived from the gonad. However, he later noted that this species is a coelomic incubator (Dartnall, 1971). Although there is some discrepancy about the portion of the incubation (Dartnall, 1969, 1971), Chia (1976) reported that *P. vivipara* is an intraovarian brooder. Thus, *P. vivipara* seems to be a viviparous species. In *Xyloplax medusiformis*, development occurs in the ovary (Rowe *et al.*, 1988). Although this species is dioecious, eggs, sperms, embryos at various stages, and juveniles are present in the ovary. Thus, this species is truly ovoviviparous. Recently, Concentricycloidea, to which *X. medusiformis* belongs, has been regarded as a member of the subphylum Asterozoa, rather than of the Crinozoa or Echinozoa (Barker *et al.*, 1986). Therefore, further studies on the developmental process in *X. medusiformis* may provide important informations about true ovoviviparity in Asterozoa. Thus, previous descriptions suggest that the last mentioned five species are truly viviparous. The present study has shown that development of *Asterina pseudoexigua pacifica* commences and proceeds throughout metamorphosis within the gonad; the resulting juveniles are released from the gonopore. Thus, *A. pseudoexigua pacifica* is the sixth species of truly viviparous echinoderms.

Embryos of *A. pseudoexigua pacifica* have no tissue connection with the adult. Rather, the nutritional requirements of the embryos in these viviparous echinoderms seem to be supplied by nutrient reserves in the egg. Embryos of the bursal brooding ophiuroids, *Amphipholis japonica* and *Amphipholis squamata*, do have an organic connection with the bursal wall, and these ophiuroids were thought to be viviparous, the larva being brooded in the bursa and nourished by the adult (Murakami, 1940; Fell, 1946). But the tissue connection now appears to be a supporting structure, as had been suggested by Fell (1946).

Both male and female elements mature simultaneously in the gonads of *A. pseudoexigua pacifica*. Therefore, this species is spatially hermaphroditic. Spatial hermaphroditism has been reported in a few asterinid species: *Asterina gibbosa* (Cuénot, 1898; Bacci, 1949, 1951; Delavault, 1966), *Asterina minor* (Komatsu *et al.*, 1979) and *Asterina phylactica* (Emson and Crump, 1979), suggesting that spatial hermaphroditism is not rare in this genus.

Chia (1976) mentioned that *P. vivipara* is probably self-fertilizing. *Asterina minor* is self-fertilizing (Komatsu *et al.*, 1979). Fertilization in Echinodermata generally occurs in seawater, but Mortensen (1921) reported that ripe eggs of a true viviparous ophiuroid, *O. hexactis*, are fertilized in the ovary. Internal fertilization has not been described previously in any asteroid species. Although we have not observed natural self-fertilization in *A. pseudoexigua pacifica*, the development was initiated in the hermaphroditic gonads by injecting 1-methyladenine into the coelomic cavity of the adult in June. Therefore, we assume that self-fertilization takes place by the sperm of the same individual (Komatsu, unpubl.). The gonads of *A. pseudoexigua pacifica* have mature sperms and full-grown ova, simultaneously, in early June. The gonads contain many embryos or juveniles from mid-June to mid-July. These facts suggest that self-fertilization takes place internally.

Brooding occurs in many asteroid species, but the brooding habits differ with species (Feder and Christen, 1966; Hayashi, 1972). The protective location of the embryos varies: underneath the disk in *Leptasterias ochotensis similispinis* (Hayashi, 1943; Kubo, 1951); in a brooding chamber beneath the disk in *Henricia sanguinolenta* (Sars, 1844; Masterman, 1902), *Henricia tumida* (Hayashi, 1940), and *Leptasterias hexactis* (Osterud, 1918; Chia, 1966); in the nidamental chambers that are formed by interlocking spines at the arm bases in *Odinella nutrix* (Fisher, 1940); among the bases of the outspread spinelets of the dorsal paxillae in *Ctenodiscus australis* (Lieberkind, 1926); in the nidamental cavity between the aboral body wall and the supradorsal membrane in *Pteraster militaris* (Koren and Danielssen, 1857) and *Pteraster obscurus* (D'yakonov, 1968); and in the stomach in *Leptasterias groenlandeca* (Lieberkind, 1920; Fisher, 1930).

Ovoviviparity may be considered to be a type of brooding. However, a definite difference exists between the viviparity and brooding outside the gonad. In asteroids, maturation of ova takes place during their release from the gonads. On the other hand, ova of the viviparous asteroid should mature within the gonads. Furthermore, many physiological changes must have occurred in the embryos during the change in adaptation, from the seawater environment, to the intraovarian circumstances. Viviparity is thus a very unique and specialized way of protecting the embryos in asteroids.

As adaptations for protecting offspring, specific methods and sites have been developed in each species during evolution. The ovoviviparity of *A. pseudoexigua pacifica* should reflect a period of evolution unsuitable for free larval life, during which the present species evolved.

Observations on the development of truly viviparous echinoderms are limited. Even in *O. hexactis*, the development of which has been thoroughly reported, the process of metamorphosis is unknown (Mortensen, 1921). Thus, the entire process of development of a true viviparous species is reported for the first time in the present study. Eggs of *A. pseudoexigua pacifica* are 450 μm in diameter, more than twice the size of those of *P. vivipara* (Chia, 1976; 150 μm) or of *O. hexactis* (Mortensen, 1921; 200 μm). *Patiriella vivipara* has no larval stage. Development of *X. medusiformis* is direct, and embryos are present in the gonad (Rowe *et al.*, 1988). In *L. clarki*, egg diameters range from 240 to 404 μm, and development proceeds, within the ovary, to tentacled juveniles through pentactula larvae (Everingham, 1961; cited from McEuen, 1987). *Ophionotus hexactis* has an ophiopluteus larva that is rudimentary, because the arms develop poorly and the anus is not open. The larva of *A. pseudoexigua pacifica* is a pear-shaped brachiolaria, similar to that of asteroids with direct development. Differences in the egg and the mode of development in these viviparous echinoderm species may indicate that ovoviviparity in echinoderms is a result of convergence.

Development of *A. pseudoexigua pacifica* is direct, with only a brachiolaria stage and without a bipinnaria. Direct development has been reported in other *Asterina* species: *A. batheri, A. burtoni, A. coronata japonica, A. exigua, A. gibbosa,* and *A. minor* (MacBride, 1896; Mortensen, 1921; James, 1972; Komatsu, 1975; Kano and Komatsu, 1978; Komatsu *et al.*, 1979). The eggs of these asterinids are yolky and large in diameter. The morphology of the larva of *A. pseudoexigua pacifica* resembles that of other asterinid species having direct development, especially *A. coronata japonica*. Brachiolariae of *A. pseudoexigua pacifica* and *A. coronata japonica* have short brachiolar arms and a poorly developed central sucker. All asterinids undergoing direct development have a pelagic larval phase, except for *A. exigua, A. gibbosa,* and *A. minor*. Although the larvae of *A. pseudoexigua pacifica* remain in the gonad throughout their development, they have cilia on the body surface and can swim in seawater when removed from the ovary. Furthermore, the larvae continue developing in seawater (Komatsu, unpubl.). Hence, the larva of the ancestor of *A. pseudoexigua pacifica* may have been free-swimming in seawater like the larvae of some other asterinids. Although *A. pseudoexigua pacifica* is ovoviviparous, it has the same developmental type, egg, and brachiolaria as other non-viviparous asteroids. This suggests that ovoviviparity in *A. pseudoexigua pacifica* evolved recently from the direct type with free-swimming brachiolaria.

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Roles of the Polar Cytoplasmic Region in Meiotic Divisions in Oocytes of the Sea Cucumber, *Holothuria leucospilota*

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Abstract. The sea cucumber oocyte has a marked cytoplasmic protrusion at its presumptive animal pole. The role of this cytoplasmic region (called the "pole" in this paper) in meiotic divisions was investigated. With maturation, the germinal vesicle (GV) migrates to the pole and breaks down. When the migration of the GV was impeded by compression, a pair of asters developed in the cytoplasmic region at the pole. A meiotic spindle formed when these two asters united with the nucleoplasmic area after breakdown of the GV. The origin of these asters was then examined by transecting oocytes microsurgically. Upon maturation, the fragments containing the pole, but lacking the GV, developed a pair of asters in the cytoplasmic region at the pole. Fragments containing both the pole and the GV formed a meiotic spindle. However, no asters formed in fragments lacking the pole (either containing or lacking the GV). The results demonstrate that the pair of asters are the organizing centers of the spindle, and that they are derived from the pole, indicating that the centrosome(s) resides in the pole of the oocyte.

Introduction

Prophase-arrested oocytes of sea cucumbers have a conspicuous cytoplasmic protrusion (Gerould, 1896; Ohshima, 1921; Inaba, 1930; Maruyama, 1980; Smiley and Cloney, 1985). With maturation, polar bodies form by a pinching-off of the protrusion (Maruyama, 1980, 1981, 1985). Thus, the protrusion is a marker of the presumptive animal pole. Similar protrusions have been described in oocytes of sea lilies (Holland *et al.*, 1975) and of some sea urchins (Jenkinson, 1911; Lindahl, 1932; Monné, 1946).

The protrusion in sea cucumber oocytes contains fibrillae (Ohshima, 1921, 1925; Inaba, 1930), and in the growing oocytes of *Holothuria monacaria,* the fibrillar structure is reported to originate from a hematoxylin-stained oval body (Oka, 1940). Electron microscopy revealed that an array of microtubules extend from the protrusion to the GV in oocytes of another sea cucumber, *Stichopus californicus* (Smiley and Cloney, 1985). In starfish oocytes, a pair of asters and centrosomes (called "premeiotic asters") are pre-existing and associated with the cell surface at the presumptive animal pole (Wilson and Mathews, 1895; Schroeder and Otto, 1984; Schroeder, 1985a, b; Picard *et al.*, 1988).

The present study was initiated to elucidate the role of the cytoplasmic region of the protrusion in meiotic divisions in oocytes of the sea cucumber, *Holothuria leucospilota.* I found that a pair of asters responsible for meiotic spindle formation are derived from the protrusion of the prophase-arrested oocyte. The cytoplasmic region of the protrusion in oocytes will be referred to, in this paper, as the "pole"; it corresponds, in terms of classical embryology, to the presumptive animal pole.

Materials and Methods

Oocytes

Adult specimens of *H. leucospilota* were collected in the vicinity of the Seto Marine Biological Laboratory (Shirahama, Wakayama prefecture) from July through August. Ovaries and isolated oocytes were prepared as described previously (Maruyama, 1980, 1985). Oocytes within, or isolated from, the ovaries were immature, arrested at prophase-I.

Observation of maturation process

Oocytes in ovaries were induced to mature by treatment with radial nerve extracts, as described previously (Ma-

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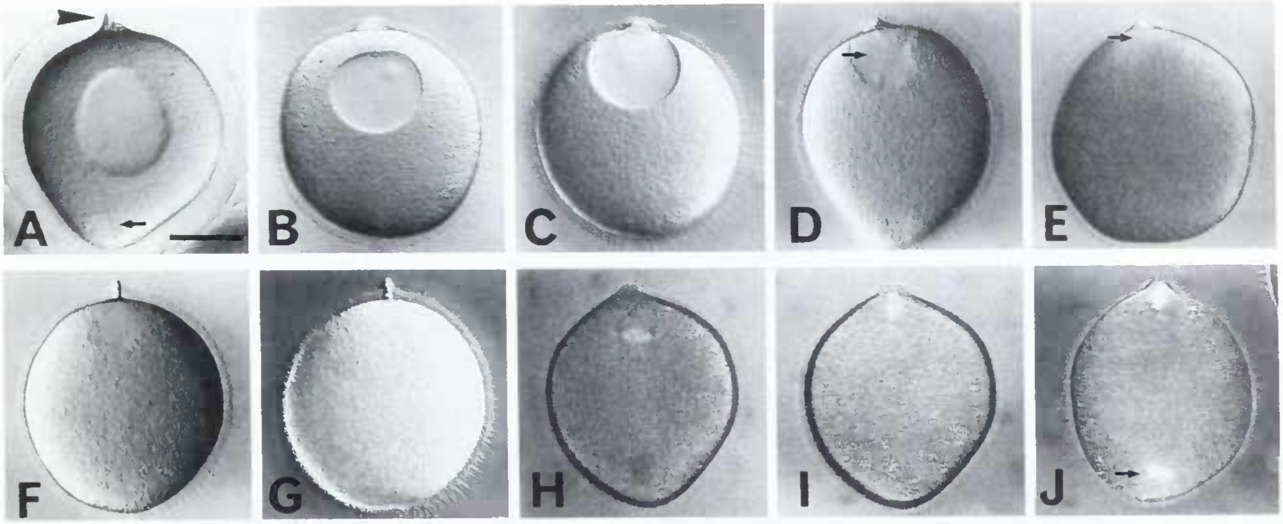

Figure 1. Maturation in intact oocytes of *Holothuria leucospilota*. Oocytes were induced to mature by treatment with radial nerve extracts and were observed with Nomarski (A–G) and polarizing (H–J) microscopes. A: A prophase-arrested oocyte with follicle cells. A cytoplasmic protrusion (top, indicated by an arrow-head) and a cytoplasmic islet (bottom, indicated by an arrow) are seen. B: 14 min. Germinal vesicle (GV) migration. C: 17 min. D: 27 min. Breakdown of GV. The arrow (also in E) indicates chromosomes. E: 50 min. Metaphase. F: 65 min. Telophase. The first polar body is forming from the elongated protrusion. G: 100 min. The second polar body is forming. H: A newly formed meiotic spindle in an oocyte at 30 min. I: The meiotic spindle at 36 min (the same oocyte as in H). The spindle attaches to the pole with one end. J: The meiotic spindle (top) at 57 min. The birefringent body (arrow at the bottom) is the clear spot. Temperature: 27–28°C. Bar in A: 50 μm (for A–J).

ruyama, 1985). Eight to ten minutes later, the oocytes were isolated, washed once, and used for observations. Maturation could also be induced by treating isolated oocytes with dithiothreitol (DTT: Wako Pure Chem. Comp. Ltd., Osaka) dissolved in seawater at a final concentration of 1 m*M* (Maruyama, 1980). At appropriate intervals, maturing oocytes were pipetted onto a glass slide and covered with a cover slip. A pair of glass rod spacers (about 200 μm thick), placed between the cover slip and the glass slide, permitted the observation of intact oocytes without compression. The specimens were observed with a light microscope through Nomarski or polarization optics.

In this paper, "developmental time" is reckoned in minutes from the start of induction of maturation. Manipulations and observations were made at room temperature (24–30°C).

Compression of oocytes to suppress migration of germinal vesicles

Isolated oocytes were induced to mature by treatment with seawater containing 1 m*M* DTT. Five to twenty minutes after the start of induction, a small number of oocytes at GV-containing stages were transferred to a drop of seawater containing 1 m*M* DTT on a glass slide; the oocytes were then compressed under a fragment of cover slip, spaced by three pieces of glass rod (50–70 μm thick). Once the drop of seawater under the cover slip had been sealed with silicone oil (SH-1107, Nakarai Chemicals Ltd., Kyoto), the specimen could be observed successively through Nomarski optics. The oocytes examined were those in which the main axis (defined by the pole) was parallel to the glass surfaces. For each oocyte, the "distance" between the pole and the nucleus—defined by the shortest length from the pole to the GV, either just before or after its breakdown—was measured with an ocular scale calibrated with a stage micrometer. The largest diameters of some oocytes and their GVs were measured. When the oocytes were under compression, the cell and GV diameters were, respectively, about 1.4–1.5 times and 1.1–1.2 times larger than those of uncompressed oocytes.

Microsurgical operation of oocytes

Isolated oocytes were transected into two fragments, under a dissecting microscope, with a glass microneedle (*cf.* Maruyama *et al.*, 1986). The transections were made at various locations with respect to the pole, as depicted in Figure 4. The fragments resulting from this surgery were treated with seawater containing 1 m*M* DTT to induce maturation. Five to twenty minutes later, they were transferred onto glass slides and observed as described above.

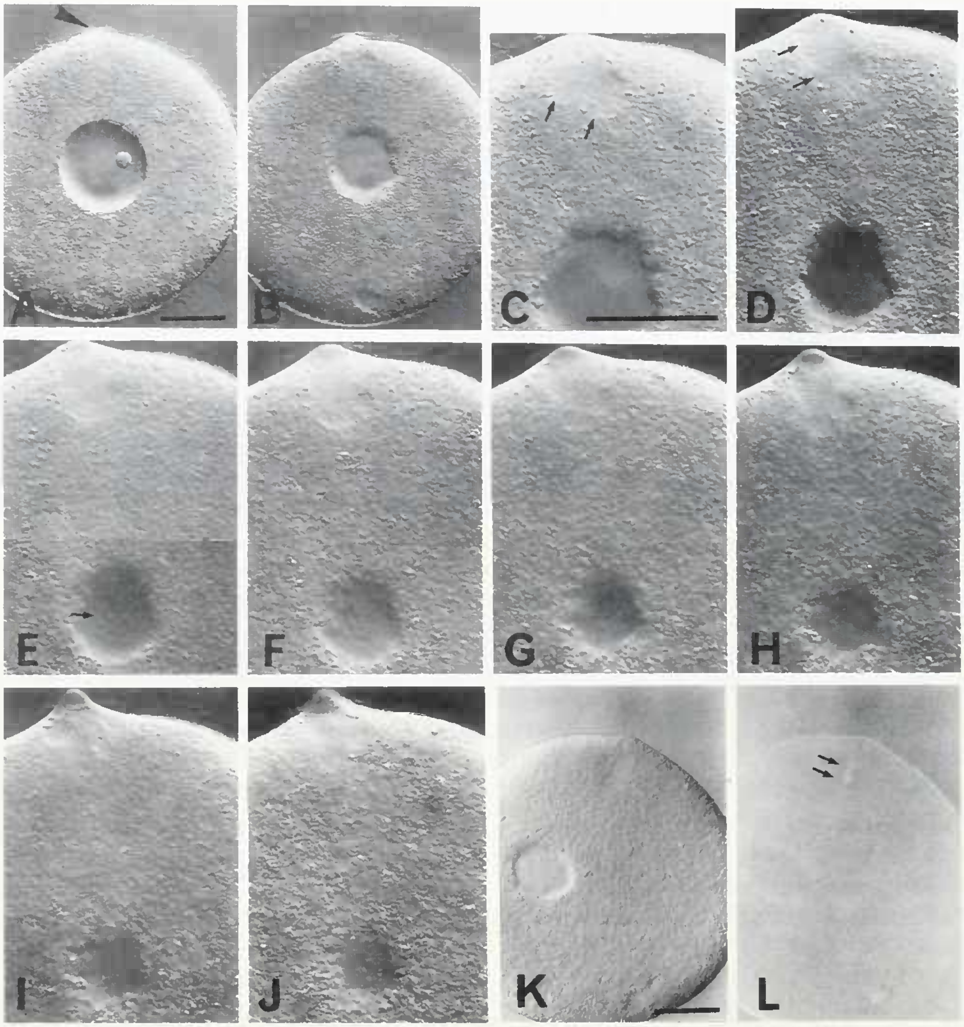

Figure 2. Dissociation of spindle organization-centers from the nucleus in oocytes compressed before GV migration (at 10 min of maturation). The oocytes were successively observed (A–J and K–L illustrate different oocytes). A: 23 min. The arrow-head points to the pole. B, C: 33 min. The GV has broken down. The distance between the GV and the pole is 72 μm. A pair of asters (indicated by arrows; also in D) at the pole (top), and the clear spot (bottom) at the antipole, are seen. D: 48 min. E: 58 min. The arrow points to the chromosomes. F: 63 min. G: 73 min. H: 83 min. I: 88 min. J: 98 min. K: Another oocyte at 40 min. A pair of asters are some distance from the nucleoplasmic area. L: The same oocyte as K, observed under a polarizing microscope. The pair of asters show a spindle-like figure. A birefringent body (bottom-right) is the clear spot. Bars: 50 μm (in A for A–B, in C for C–J, and in K for K–L). Temperature: 24–27°C.

Results

Meiotic events in intact oocytes

In prophase-arrested oocytes, the GV was located centrally or slightly off-center toward the pole (Fig. 1A). The GV began to migrate toward the pole 11–15 min after the start of maturation (27–29°C), and finally became associated with the pole (Figs. 1B, C). Follicle cells surrounding each oocyte were detached during the migration. The GV broke down at about 20 min (Fig. 1D), and the disappearance of its membrane left a transparent nucleoplasmic area adjacent to the pole. Chromosomes and two small birefringent asters appeared there, and a spindle with two astral foci formed (Fig. 1H). Chromosomes were aligned to form a metaphase plate (Fig. 1E).

The newly formed meiotic spindle was oriented obliquely or vertically with regard to the main axis of the oocyte, defined by the pole (Fig. 1H). Through its rotation at 30–40 min (Fig. 1I), the spindle was "attached" to the pole with one end (Fig. 1J). With this attachment, cytoplasmic granules were displaced from the polar region

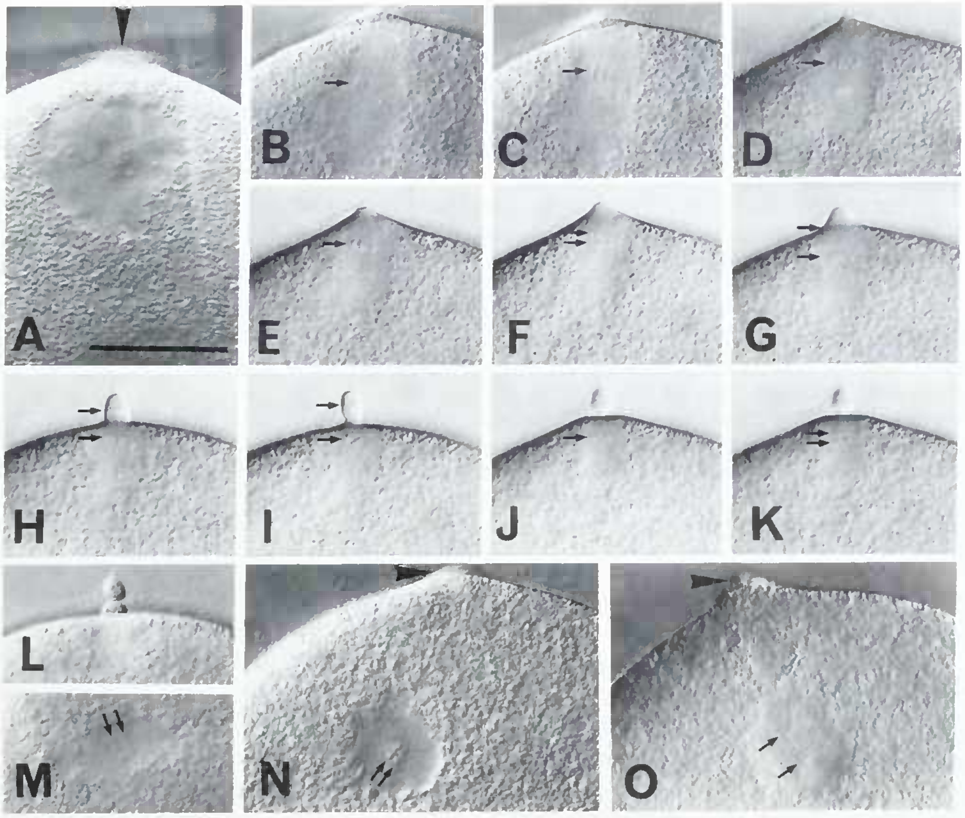

Figure 3. Meiotic spindle formation or polar body formation in an oocyte compressed after migration of the GV (at 20 min, A–L), and in oocytes compressed from the beginning of GV migration onward (at 15 min, M–O). Maturation was induced either with 1 m*M* DTT (A–L, M and O) or with radial nerve extracts (N). A: 25 min. The arrow-head points to the pole. B: 35 min. Metaphase. The arrow points to the chromosomes (also in C–K). C: 45 min. D: 55 min. E: 65 min. F: 70 min. Early anaphase. G: 74 min. Late anaphase. H: 75 min. I: 76 min. Telophase. The first polar body is forming. J: 119 min. Second metaphase. K: 123 min. Second anaphase. L: 128 min. The second polar body is forming. M: Anaphase chromosomes (arrows) in an oocyte at 70 min. The distance between the GV and the pole was 72 μm. N: Anaphase chromosomes (arrows) in an oocyte at 66 min. The distance between the GV and the pole was 32 μm. The arrow-head points to the pole (also in O). O: Telophase chromosomes (arrows) in an oocyte at 67 min. The distance between the GV and the pole was 36 μm. Temperature: 24–27°C. Bar in A: 50 μm (for A–O).

(Fig. 1E). Then the pole elongated radially, and one set of chromosomes passed through the base of the pole (Fig. 1F). The pole was constricted at its base to form the first polar body. The second polar body was formed just beneath the first (Fig. 1G, and also see Fig. 3I–L).

A transparent cytoplasmic islet ("clear spot") occurs in the cytoplasm of maturing oocytes at the side opposite that of the pole (*cf.* Fig. 2B; Maruyama, 1981). Observations with polarization optics revealed that the spot is birefringent (Fig. 1H, J). By careful observation, the spot could be traced back to the prophase-arrested oocytes (Fig. 1A, arrow).

Oocytes treated with radial nerve extracts and those treated with DTT matured similarly and had similar morphological features, as shown previously (Maruyama, 1980, 1985).

Dissociation of meiotic spindle-organizing centers from the nucleus

Maturing oocytes at GV-containing stages were subjected to compression under a cover slip to impede the migration of the GV. When oocytes at 5 or 10 min (*i.e.*, before GV migration) were compressed, the GV broke down at an ectopic site apart from the pole (Fig. 2A, B), and the nucleoplasmic area thus formed remained there (Fig. 2). A pair of asters appeared at the pole just after GV breakdown (Fig. 2B, C); they showed only weak birefringence under a polarization microscope (Fig. 2L). At first, they moved away from the pole for a short distance, and then returned to the pole (Fig. 2D). As the asters moved to the pole, cytoplasmic granules contained in the polar region were displaced (Fig. 2D–J). Surprisingly,

Table I

Occurrence of meiotic spindles and polar bodies in oocytes under compression[1]

Initiation of compression	No. of oocytes	Distance[2]	Meiotic spindles[3] +	Meiotic spindles[3] −	Polar body[4] formation
Before migration	31 (9)	98 [70–148]	2 (1)	29 (8)	0 (0)
During migration	34 (13)	35 [12–110]	31 (12)	3 (1)	17 (6)
After migration	26 (9)	5 [0–28]	26 (9)	0 (0)	22 (8)

[1] Oocytes were induced to mature by treatment with 1 m*M* DTT or radial nerve extracts, compressed, and observed successively (24–27°C). Results from the latter were given in parentheses as fractions of cases.

[2] The "Distance" is the mean value with its range (brackets) in μm between the pole and the nucleus, measured for each oocyte at a time just before or after breakdown of the germinal vesicle.

[3] The meiotic spindle formation was examined through successive observations from about 30 min up to about 70 min. +, spindle formed; −, did not.

[4] Polar body formation was examined through further observations of each oocyte up to 96–190 min.

chromosomes appearing in the nucleoplasmic area remained there without forming karyokinetic figures (Fig. 2E–J). Thus, most (29/31) of the oocytes failed to form meiotic spindles (Table I). Nevertheless, a small fraction (3/29) of these oocytes did form a metaphase-like chromosome configuration at a later time (at 120–140 min). In the remaining 2 out of 31 oocytes, metaphase to anaphase figures were detected at 50–70 or 55–80 min (Table I).

Maturing oocytes at 11–16 min (*i.e.*, during GV migration) were then compressed (Table I). The GV broke down near the pole (*cf.* Fig. 3N, O). A pair of asters were first detected, either at the margin of the nucleoplasmic area facing the pole, or in the cytoplasm adjacent to the pole. The asters then moved into the nucleoplasmic area, and chromosomes in the nucleoplasmic area were aligned to form a metaphase plate at 30–50 min. Most (31/34) of the oocytes formed meiotic spindles (Table I).

When oocytes were compressed as late as 19–20 min (*i.e.*, after GV migration), the nucleoplasmic area formed at the pole (Fig. 3A), and in these cases, meiotic spindles formed (Fig. 3B–L, Table I). In summary, meiotic spindle formation in compressed oocytes is apparently dependent upon the time of compression.

Polar bodies formed in oocytes under compression (Table I). This occurred in cases where the metaphase spindle moved, and "attached" to the pole (Fig. 3B–E). The first polar body formed by a pinching-off of the protrusion at its base (Figs. 3F–I), and the second polar body formed just beneath the first (Figs. 3J–L), as in uncompressed oocytes. In contrast, when the metaphase spindle failed to move and attach to the pole, polar bodies failed to form, but anaphase movement of chromosomes still occurred (Fig. 3M–O).

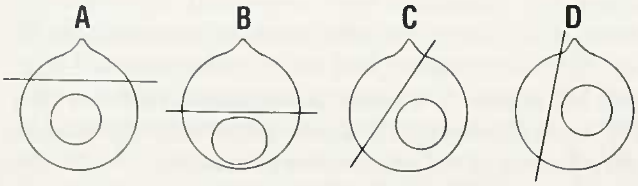

Figure 4. Transection of prophase-arrested oocytes. The oocytes were transected at one of the planes indicated (A–D) with reference to the protrusion marking the presumptive animal pole. On transection, the GV ends up in one of the daughter fragments.

As shown in Table I, spindle and polar body formation failed, even in those compressed oocytes that had been obtained from ovaries stimulated with radial nerve extracts. Thus, such failures are not due to the effects of DTT used for maturation. The distance between the pole and the nucleus at the time of GV breakdown may be important for the successful formation of the meiotic spindle and polar bodies in oocytes under compression (Table I).

The results show that a pair of asters develop in the cytoplasmic region at the pole after GV breakdown, and that the asters are required for organizing the meiotic spindle.

The pair of asters required for meiotic spindle formation are derived from the pole of the prophase-arrested oocyte

Prophase-arrested oocytes were microsurgically transected (Fig. 4), and the GV-enucleate fragments, whether containing the pole or not, were treated to induce maturation and observed (Table II).

Fragments containing the pole developed a pair of asters in the cytoplasmic region at the pole (Fig. 5A–D), whereas fragments lacking the pole did not. Two asters were invariably observed at 20–40 min (Table II); later (at 80 min or more), however, about half of the fragments had four asters. This shows that in fragments lacking the contents of the GV, each of the asters had split into two. Autonomous replication of centrosomes has been shown in starfish oocytes deprived of GV materials (Picard *et* *al.*, 1988) and in enucleated sea urchin embryos (Lorch, 1952; Sluder *et al.*, 1986).

Fragments with a GV, but lacking the pole, were obtained and induced to mature (12 from the bisection B and 11 from the bisection C in Fig. 4). The GV still broke down, but the asters did not form. The chromosomes did not form karyokinetic figures and remained in a single cluster in the nucleoplasmic area (Fig. 5E). On the other hand, most (8 out of 9) fragments with both a GV and a pole region (produced by transecting prophase-arrested oocytes through plane D, Fig. 4) exhibited karyokinetic figures showing metaphase- or anaphase-chromosomal configurations (Fig. 5F).

These results show that the pair of asters required for meiotic spindle formation are derived from the polar region of the prophase-arrested oocyte. Therefore, the organizing center(s) (centrosome) for the meiotic spindle probably resides in the pole of the prophase-arrested oocyte.

Table II

Aster formation in GV-enucleate fragments with or without the pole, obtained from prophase-arrested oocytes

Fragments[1]			No. of asters[2]		
Transection	Pole	No.	2	1	Undetected
A	+	23	22 (12)	0	1
B	+	21	21 (21)	0	0
C	+	14	14 (8)	0	0
D	−	15	0	0	15

[1] Prophase-arrested oocytes were transected in various directions (Fig. 4), and GV-enucleate fragments either containing the pole (+) or lacking the pole (−) were treated with 1 m*M* DTT and observed from about 20 min to about 40 min at room temperature (24–27°C). The fragments lacking the pole were examined further up to about 100 min.

[2] The number of asters detected is indicated. The number in parentheses indicates the fraction of cases in which the site of the pole was recognizable. In these cases, the asters appeared in a cytoplasmic region close to the pole.

Discussion

Poles as associated sites of centrosomes

The present study has revealed a pair of asters at the pole of living sea cucumber oocytes during maturation. The occurrence of fibrillar structures (Ohshima, 1921, 1925; Inaba, 1930) and microtubules (Smiley and Cloney, 1985) at the pole in prophase-arrested oocytes suggests that the asters may pre-exist in some form in immature oocytes. And Oka (1940) reported a fibrillar structure even in growing oocytes. The present study demonstrates that the organizing centers of the meiotic spindle are derived from the pole in prophase-arrested oocytes. These findings imply that the fibrillar structure at the pole is a structure

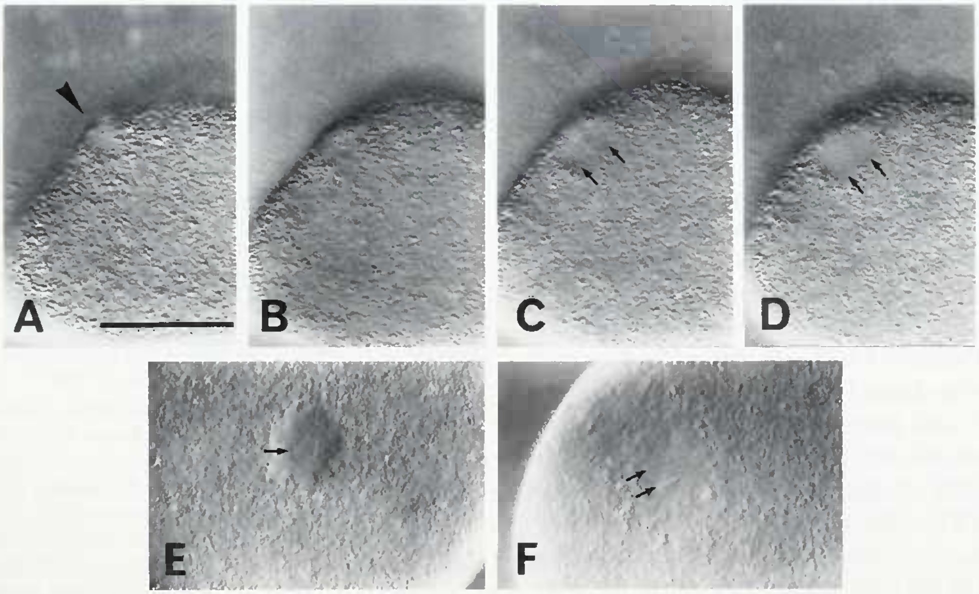

Figure 5. Aster formation in a GV-enucleate fragment with the pole (A–D), and chromosome arrangements in GV-nucleate fragments without (E), and with (F), the pole. The fragment in A–D was obtained from transection A (Fig. 4), and those in E and F were obtained from transections C and D, respectively. A: 9 min. The pole (arrow-head) looks clear. B: 15 min. C: 21 min. A pair of asters (arrows; also in D) form at the pole. D: 30 min. E: 93 min. Chromosomes (arrow) show no karyokinetic figures. F: 65 min. Anaphase chromosomes (arrow). Temperature: 24–27°C. Bar in A: 50 μm (for A–F).

similar to the pre-meiotic aster seen in starfish oocytes (Schroeder, 1985a). I conclude that the pole (*i.e.*, the presumptive animal pole) of the prophase-arrested oocyte of the sea cucumber is the site of the centrosome(s) or microtubule-organizing center(s). With maturation (perhaps, at a time prior to GV breakdown), the centrosome may change to form a pair of asters, and these asters (centrosomes) then function as the organizing centers of the meiotic spindle.

In starfish, the pre-meiotic asters are associated with the cortex at the presumptive animal pole (Schroeder and Otto, 1984, Schroeder, 1985a, b; Picard *et al.*, 1988). A similar microtubule-array containing a microtubule-organizing center occurs in association with the cell surface in GV stage oocytes of sea urchins (Boyle and Ernst, 1989). Hence, the association of the centrosome with the cell surface of the presumptive animal pole where polar bodies later form may be a characteristic common to all echinoderm oocytes.

Poles and meiotic divisions

As can be seen in Figure 1, features specific to oocytes of the sea cucumber, *Holothuria leucospilota*, are the migration of the GV to the pole and the subsequent formation of a meiotic spindle in the polar region. Because the centrosome participating in meiotic spindle formation is initially dissociated from the GV and resides at the pole, a direct outcome of the GV migration is 'restoration' of the spatial association of the centrosome(s) with the nucleus to form the meiotic spindle. The pole, embracing the centrosome (microtubule-organizing center), microtubules, and the cortex, may participate in the migration of the GV to the pole.

Nuclear migration, or spindle migration, and subsequent attachment of the spindle pole to the cortex results in unequal divisions: *e.g.*, polar body formation in surf clam oocytes (Dan and Ito, 1984; Dan and Inoué, 1987), *Chaetopterus* oocytes (Hamaguchi *et al.*, 1983) and *Crepidula* oocytes (Conklin, 1917); micromere formation in sea urchin embryos (Dan, 1979, 1984; Dan *et al.*, 1983; Schroeder, 1987); and ganglion cell formation in neuroblasts of grasshoppers (Kawamura, 1977). The present study of the meiotic divisions of sea cucumber oocytes has revealed that a meiotic spindle eventually "attaches" to a definite site—the pole—to form the polar bodies. Thus, the cortex of the pole is a site specialized for interacting with the spindle-pole aster, and for anchoring the microtubule-organizing center or the asters originating from it. The cortex or cell surface of the pole may contain local factors responsible for binding with asters or the organizing center.

Polar protrusion and clear spot as markers of the animal-vegetal axis in sea cucumber oocytes

The presence of the "clear spot," a special cytoplasmic islet, in the cytoplasm near the cell surface opposite the presumptive animal pole (Maruyama, 1981) could be traced back to prophase-arrested oocytes. With maturation, the clear spot exhibits a rather strong birefringence. Further studies are needed to define its significance in development. In any event, there are now two visible structures, the polar protrusion and clear spot, both serving as markers for the main axis (animal-vegetal axis) of the oocyte. They could be useful for analyzing localized morphogenetic determinants, as has been done in eggs and embryos of sea urchins and starfish (Maruyama *et al.*, 1985; Maruyama and Shinoda, 1990).

Acknowledgments

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Self and Non-Self Recognition in a Calcareous Sponge, *Leucandra abratsbo*

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Abstract. Discrimination between self and non-self has been shown in many demosponges, but calcareous sponges have not been studied. Allorecognition in a calcareous sponge, *Leucandra abratsbo,* was analyzed in allogeneic combination assays. Most allogeneic combinations were incompatible, and the low rate (4.8%) of allogeneic acceptances suggests an extensive polymorphism in those genes that may control allorecognition. However, histological studies of the rejection process revealed that the first reaction consisted of strong adhesion of allogeneic pieces. Thereafter, the rejection reaction that followed was accompanied by the accumulation of archeocytes in the contact region. Vigorous cytotoxic reactions occurred within this region, and the degenerated cells were probably phagocytosed by archeocytes, which suggests that they are the primary effector cells for cytotoxicity and phagocytosis. Because *L. abratsbo* is a solitary sponge, armed with protruding spicules that prevent contact of the pinacoderm with that of conspecific individuals, allorecognition may not prevent the formation of allogeneic chimeras in the natural habitat.

Introduction

The immune systems of invertebrates have interested investigators who believe that such systems might be precursors of the vertebrate immune system (Coombe *et al.*, 1984; Stoddart *et al.*, 1985). In the last decade, comprehensive studies have provided much information on sponge allorecognition (Hildemann *et al.*, 1979, 1981; Kaye and Ortiz, 1981; Curtis *et al.*, 1982; Jokiel *et al.*, 1982; Van de Vyver and Barbieux, 1983; Buscema and Van de Vyver, 1984a–c; Neigel and Schmahl, 1984; Neigel and Avise, 1985; Mukai and Shimoda, 1986; Smith and Hildemann, 1984, 1986a, b). The resulting indisputable evidence suggests that allorecognition is the rule in demosponges; alloincompatibility can be induced in most orders of the class Demospongiae.

The allogeneic reactions of demosponges, however, are remarkably variable, so a thorough understanding of sponge allorecognition has been difficult. First, allografts are rejected in some sponges, but accepted in others (Jokiel *et al.*, 1982; Buscema and Van de Vyver, 1984c). Second, the rejection reaction varies considerably from species to species. According to present information, allografts are rejected by cytotoxic reactions (Hildemann *et al.*, 1979, 1981; Buscema and Van de Vyver, 1984b; Mukai and Shimoda, 1986; Smith and Hildemann, 1986a), by the formation of a collagenous barrier (Buscema and Van de Vyver, 1984a, c), or by nonfusion (Buscema and Van de Vyver, 1984c; Mukai and Shimoda, 1986). Moreover, two or three types of rejection reactions have been observed in some species (Van de Vyver and Barbieux, 1983; Buscema and Van de Vyver, 1984c; Mukai and Shimoda, 1986) and the type of allogeneic rejection is independent of sponge phylogeny. Third, various effector cells participate in the rejection reaction. Although several effector cells including archeocytes, collencytes, lophocytes, phagocytes, and amoebocytes have been identified thus far (Van de Vyver and Buscema, 1977; Van de Vyver and Barbieux, 1983; Buscema and Van de Vyver, 1984b, c; Smith and Hildemann, 1986a), we cannot predict which of these cells actually effects rejection reactions (Smith, 1988; Van de Vyver, 1988). Furthermore, we know very little about their origins and transitions as these cells develop normally.

Calcareous sponges diverged from the ancestral sponge before the Devonian period (Hyman, 1940). The shapes and composition of their spicules are distinctly different from those of demosponges, and their allorecognition systems may also be different. In this paper allorecognition in a calcareous sponge (*Leucandra abratsbo*) is presented.

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Most allogeneic combinations were incompatible, suggesting the existence of an extensive polymorphism of histocompatibility genes in natural populations. Vigorous cytotoxic reactions by archeocytes were observed in the contact region. The ecological significance of self and nonself recognition in these sponges is discussed.

Materials and Methods

Sponges

All specimens of *Leucandra abratsbo*, a calcareous sponge with a leuconoid canal system, were collected from a raft at the Breeding Center of Aomori Prefecture in northern Japan. They were abundant in scallop-breeding baskets that were hung a few meters below the water's surface. The size of the raft is about 15 × 20 m, so the maximum distance between any two specimens is about 20 m. The sponge was easily freed from the substratum because it is upright and has a stout body. Once collected, the sponges were put in water-tight containers, brought to the Asamushi Marine Biological Laboratory, and placed immediately in running seawater where they could be maintained for more than ten days. The largest specimen was about 8 cm in length; only those larger than 4 cm were used.

Assessment of incompatibility

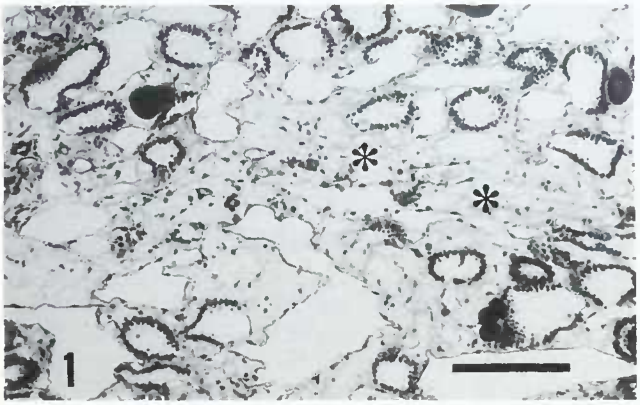
Because parabiosis experiments were not feasible with this sponge, "the allogeneic combination test" was performed as an alternative method. Sponges that had been selected for the allogeneic combination assay, were cut into slices 2 to 3 mm thick, and two sponge pieces derived from different individuals were bound together with a piece of cotton thread. The flatness of their opposed cut surfaces allowed the sponge pieces to be closely appressed, and caused the deeper sponge tissues to be in direct contact. To ensure reliability, the test was first performed with ten replicates of each sponge pair. Because all replicates of a pair showed similar allogeneic reactions, two replicates of each combination were usually performed in this study, unless otherwise mentioned. The polarity of the sponge pieces exerted no influence on their reactions in either allogeneic or autogeneic combinations. Bound sponge pieces were supplied with clean running seawater during the experiments and were as healthy as intact sponges under laboratory conditions. They regenerated the dermal layer and pinacoderm on the free cut surface during the allogeneic combination test.

The bound sponge pieces were examined daily, and most of them were distinctly rejected in four days. In preliminary experiments, five allogeneic combinations that were not rejected in four days did not reject in an additional four days. Thus, all allogeneic combinations that showed no external signs of rejection were fixed with the Bouin's solution five days after binding. To provide a time-series analysis of the rejection process, ten replicates of the same allogeneic combination were constructed from an allogeneic sponge pair. Two of these replicates were fixed daily, embedded in Palaplast, sectioned, and stained with haematoxylin and eosin.

Results

Autogeneic reactions

The fusion process was analyzed morphologically using sponge pieces in autogeneic combinations derived from one sponge specimen. One day after binding, these autogeneic sponge pieces were firmly adherent (Fig. 1). One striking feature in the contact region of such autogeneic combinations is the development of a dermal layer-like tissue between the sponge pieces. Development of this



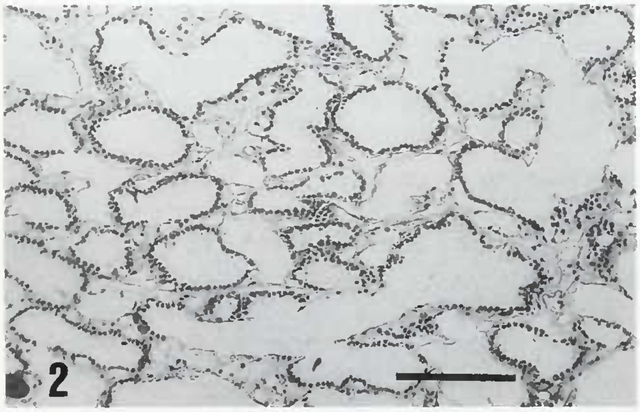
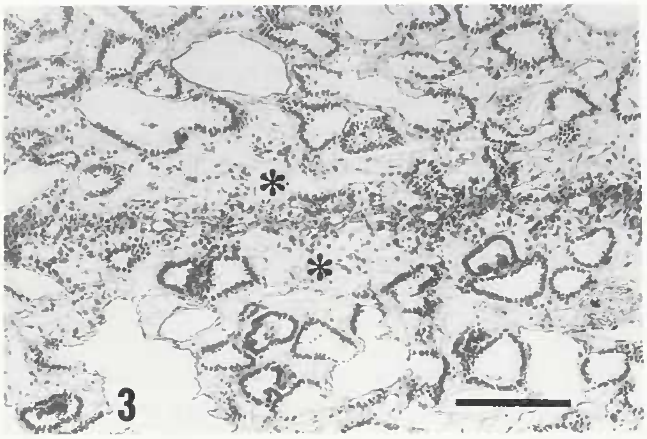


Figure 1. Fusion of a one-day autogeneic combination of *Leucandra abratsbo*. The mid-horizontal line of this photomicrograph is in the interface of the sponge pieces. The dermal layer-like tissue (asterisks) has developed in the contact region. Scale bar = 100 μm.

Figure 2. Fusion of a two-day autogeneic combination of *L. abratsbo*. Mid-horizontal line is in the interface, however, choanocyte chambers are arranged almost regularly. No dermal layer-like tissue is observable. Scale bar = 100 μm.



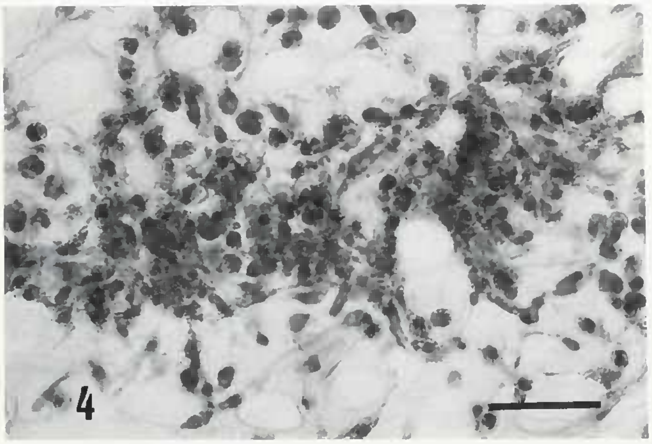


Figure 3. Rejection reaction in a one-day allogeneic combination of *Leucandra abratsbo.* Although these sponge pieces look like fusion from external observation, many archeocytes have already gathered in the contact region. The dermal layer-like tissue (asterisks) is observable on the both sides of the archeocyte accumulation. Scale bar = 100 μm.

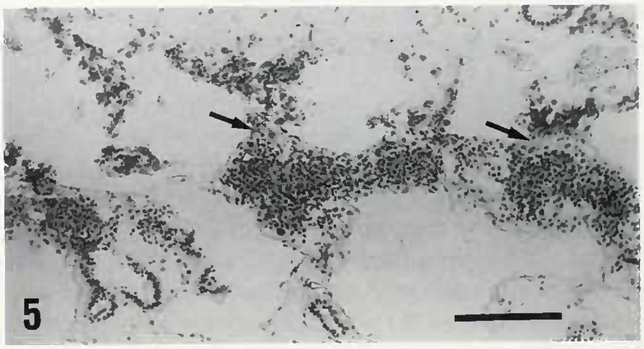
Figure 4. Archeocytes accumulated in the contact region of a one-day allogeneic combination. These archeocytes are in contact with each other, and most of them already show nuclear condensation. Scale bar = 50 μm.

tissue seems to be necessary for the fusion process, facilitating the adhesion of the sponge pieces during the early stages. Only a few archeocytes were found within the contact region.

Two days after binding, the autogeneic sponge pieces were more intimately fused than on day one, so that their external boundaries became obscure. Figure 2 shows the contact region of such sponge pieces. The dermal layer-like tissue has disappeared, and the choanocyte chambers are arranged almost regularly, with no evidence of cytotoxic or phagocytic reactions. The autogeneic sponge pieces fused rapidly, and after four days, the interface between the sponge pieces was almost undetectable microscopically; this signaled that the fusion process was complete.

Allogeneic reactions

The rejection process occurring in allogeneic combinations was studied histologically using daily samples from sets of coupled sponge pieces, each set derived from two physiologically discrete individuals of the same species. Four incompatible sponge pairs were thus observed, and they all showed a similar rejection process. The rejection process of only one allogeneic sponge pair is represented. One day after binding, allogeneic sponge pieces had adhered firmly and their pinacoderms were already fused. They are therefore difficult to distinguish from autogeneic combinations by external observation. Nevertheless, the rejection process has already begun microscopically. Figure 3 shows the contact region of a one-day allogeneic combination. As in autogeneic fusions, dermal layer-like tissue has developed in the contact region, and the sponge pieces are firmly adhered. In contrast to autogeneic fusion, archeocyte accumulations are already visible in the contact region (Fig. 4, an enlargement). The archeocytes are congregated and in close contact with each other. Within this



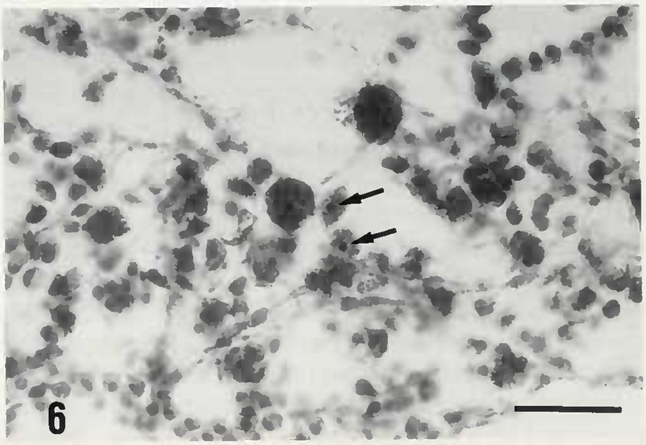
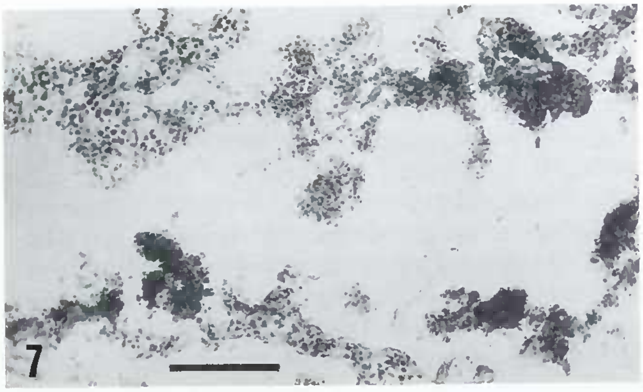


Figure 5. Rejection reaction in a two-day allogeneic combination of *Leucandra abratsbo.* Extensive degeneration of the archeocyte accumulation is shown. These sponge pieces are splitting off (arrows). Scale bar = 100 μm.

Figure 6. Phagocytes and archeocytes with conspicuous nuclear condensation (arrows) in a two-day allogeneic combination. Scale bar = 50 μm.



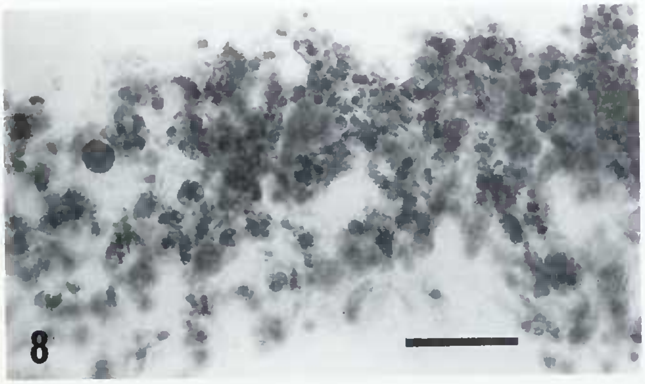
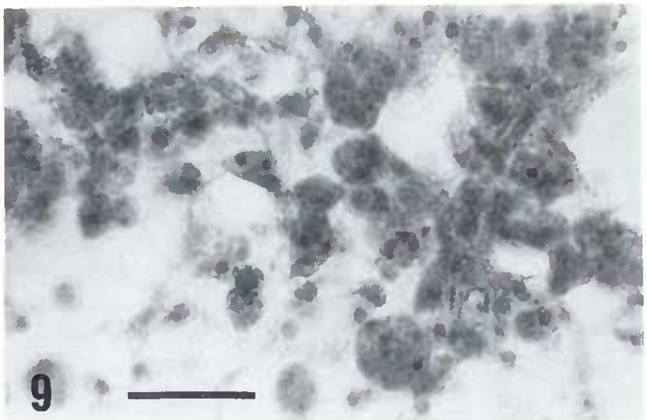


Figure 7. Rejection reaction in a four-day allogeneic combination of *Leucandra abratsbo*. The aggregates of the degenerated archeocytes have split off. Scale bar = 100 μm.

Figure 8. A large aggregate of degenerated cells in a four-day allogeneic combination. Scale bar = 50 μm.



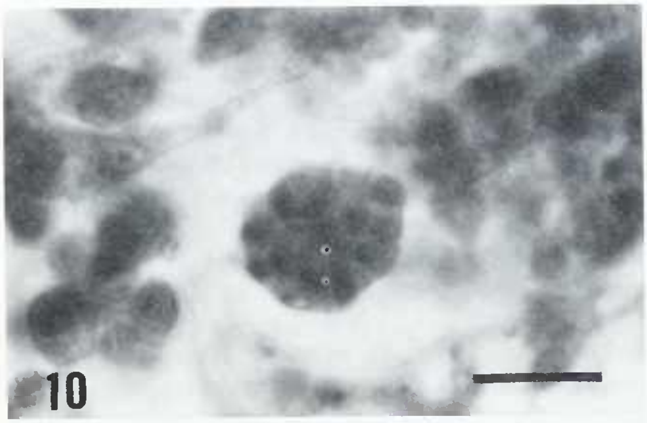


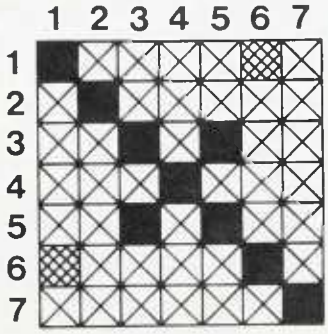
Figure 9. Many phagocytes and degenerated cells in a four-day allogeneic combination. Scale bar = 50 μm.

Figure 10. A large phagocyte that has engulfed more than ten degenerated cells. Scale bar = 10 μm.

cell accumulation, cytotoxic reactions are evident, because numerous cells show degenerative nuclear condensation. The cytotoxic reaction mediated by the archeocytes apparently begins soon after they accumulate and make cell contact.

Two days after the allogeneic sponge pieces had been bound, external signs of rejection are already evident. The fused pinacoderm begins to break along the boundary between the sponge pieces. Figure 5 shows massive accumulations of archeocytes in the contact region. Within this cell accumulation, tissue degeneration is obvious, and the sponge pieces begin to split off (Fig. 6, enlargement). Nuclear condensation is clearly visible in the degenerated cells. Phagocytosis is already discernible, and phagocytes that had engulfed several degenerated cells can be seen.

Four days after binding, extensive necrotic tissue (about 0.5 mm thick) is visible between allogeneic sponge pieces. Because it has become frail, the sponge pieces fall apart if the binding thread is removed (Fig. 7). The necrosis is, however, limited to the contact region, and the sponge tissues external to it show no degenerative signs. Thus, archeocytes, at least at four days, had apparently not invaded far into the allogeneic tissues. In the necrotic cell masses, nuclear condensation is evident in most of the cells, and cell lysis prevails (Fig. 8). The numbers of phagocytotic figures have increased considerably, and Figure 9 shows such phagocytosis in the contact region. Figure 10 reveals a phagocyte that has engulfed more than ten



	1	2	3	4	5	6	7
1	■	☒	☒	☒	☒	▩	☒
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5	☒	☒	■	☒	■	☒	☒
6	▩	☒	☒	☒	☒	■	☒
7	☒	☒	☒	☒	☒	☒	■

Figure 11. Reactions of allogeneic and autogeneic combinations between the seven individuals of *Leucandra abratsbo*. ☒, allogeneic rejection; ▩, weak rejection; ■, autogeneic fusion or allogeneic acceptance.

Table I

Allogeneic reactions in Leucandra abratsbo

	Number of combinations scored	%
Acceptance	6	4.8
Weak rejection	9	7.1
Rejection	112	88.2
Total	127	100.1

degenerated cells. Later, these sponge pieces were completely disjoined, and regeneration of pinacoderm on the re-exposed surface was observed.

Patterns and frequencies of allorecognition

Figure 11 represents an example of autogeneic and allogeneic combinations among seven individuals. All the combinations were tested twice and gave similar results. One acceptance and one weak rejection was found among the allogeneic combinations, but all of the other allogeneic combinations rejected vigorously. Seven autogeneic combinations fused completely.

Table I shows the cumulated results of 127 allogeneic combinations. About 5% of the combinations were accepted, while the others were incompatible. Histological examination of the allogeneic acceptances revealed no rejection reactions; neither archeocyte accumulation nor cytotoxic reactions were observed at the interface of the accepted sponge pieces. Accordingly, they were indistinguishable from autogeneic fusions. In about 7% of the allogeneic combinations, the sponge pieces rejected, but weakly. These weak rejections were hardly distinguishable from the allogeneic acceptances by external observation, because the fused pinacoderm was not broken. Nevertheless, histological examination revealed distinct rejection reactions in the contact region.

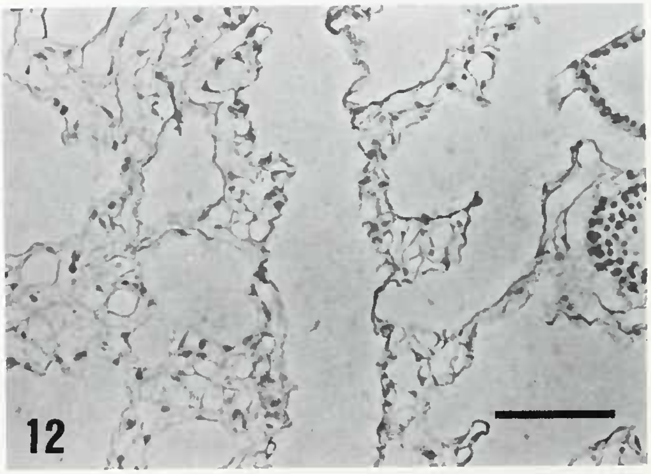
Parabiosis experiments

Parabiosis experiments, in which two sponge pieces are touching surface-to-surface, were tried. Figure 12 shows the result of an autogeneic parabiosis, and Figure 13 shows an allogeneic combination, both five days after binding. Both figures show a wide gap between the sponge surfaces because the densely protruding spicules, which have been dissolved in the Bouin's solution, prevented contact between the opposing pinacoderms. Obviously, no fusion or rejection reaction has occurred in these sponge pieces.

Discussion

Alloincompatibility in a calcareous sponge, *Leucandra abratsbo,* is shown for the first time in this report. These results suggest an extensive polymorphism of histocompatibility genes, because most individuals are alloincompatible. Obviously, further studies on other calcareous sponges are necessary to determine whether allorecognition specificity is a general phenomenon in the class Calcarea.

In this calcareous sponge, allogeneic pieces were rejected by cytotoxic reactions. Neither collagen deposition (chronic rejection) nor nonfusion was observed, although they have been shown in demosponges (Buscema and Van de Vyver, 1984a–c; Mukai and Shimoda, 1986). Many archeocytes accumulated in the contact region of allogeneic combinations of this calcareous sponge; mesohyl cell accumulations have been observed in many demosponges (reviewed by Smith, 1988). Direct contact between



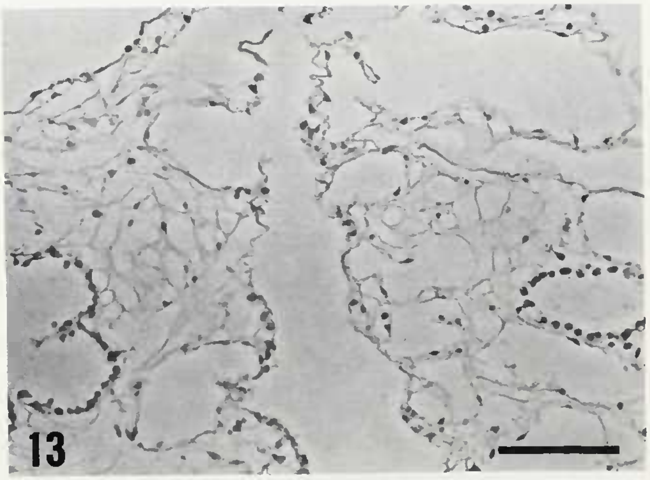


Figure 12. A parabiosis experiment of autogeneic sponge pieces five days after binding. They have not fused, and there is a wide space between their opposing pinacoderms. Calcareous spicules that prevented contact of the sponge surfaces have been dissolved in the Bouin's solution. Scale bar = 25 μm.

Figure 13. A parabiosis experiment of allogeneic sponge pieces five days after binding. There is no sign of a rejection reaction. Scale bar = 25 μm.

archeocytes is most probably necessary for the cytotoxic reaction to be triggered, because it occurred only within cell accumulations in which archeocytes were in close contact with each other. In demosponges, the necessity for contact between mesohyl cells has been suggested (Bigger *et al.*, 1981), but the involvement of diffusible substances is also plausible (Smith and Hildemann, 1986a, b). Until now, there has been no evidence that archeocytes selectively come into contact with allogeneic cells. To answer this question, *in vitro* studies may be helpful. In a solitary ascidian, *Halocynthia roretzi*, Fuke (1980) showed that the cytotoxic reaction between allogeneic coelomocytes *in vitro*, termed the "contact reaction," occurs after close contact between allogeneic cells.

Bound sponge pieces of *L. abratsbo* adhered firmly within 24 h in allogeneic combinations, as well as in autogeneic ones. By this time, the dermal layer-like tissue has developed in the contact region, and this intervening tissue may play an important role in the adhesion of sponge pieces. Because this dermal layer-like tissue formed similarly in allogeneic and autogeneic combinations, its formation is not an allogeneic reaction but more likely a regenerative event induced by the exposure of inner tissues to the exterior. Indeed, the composition of the dermal layer-like tissue was similar to that of the dermal layer that regenerated on the reverse side of the sponge pieces. Pinacoderm was formed on the surface of the dermal layer-like tissue after the sponge pieces were disunited. In autogeneic fusions, however, it disappeared from the contact region within a few days. Therefore, the dermal layer-like tissue is conceivably a regenerated dermal layer in the contact region.

About 95% of the allogeneic combinations of *L. abratsbo* were incompatible in natural populations collected from a raft (15 × 20 m). This high rate of alloincompatibility reflects extensive dispersion of sponge larvae. This calcareous sponge released amphiblastula larvae in the morning, and they swam actively, settled, and metamorphosed on the substratum (Amano, in prep.). Before settlement, they crawled about on the substratum for several hours. In demosponges, also, larval release is controlled by light (Amano, 1986, 1988); phototaxis and geotaxis enable the swimming larvae to settle in a suitable site, often at a considerable distance (Bergquist *et al.*, 1970). Twenty-four hours of swimming and transport by water currents are probably sufficient for the released amphiblastula larvae to be dispersed beyond the limits of the raft (15 × 20 m). Therefore, some specimens used in this study may be kin, and allogeneic combinations of the sponges with kinship may result in fusion. Not knowing the genealogies of the tested specimens, however, we cannot know whether allogeneic acceptances necessarily imply genetic identity of the combined individuals of *L. abratsbo* (Grosberg, 1988).

Because *L. abratsbo* is densely covered with protruding stout spicules, they prevented sponge pieces from touching each other when parabiosis experiments were tried. Without immediate contact between their opposing pinacoderms, the sponge pieces did not fuse, nor were there rejection reactions even in autogeneic or allogeneic individuals. Therefore, allorecognition in this sponge is not required to avoid fusion and the formation of allogeneic chimeras in nature. But if it is so, why has this sponge developed a recognition system that can be revealed only in the laboratory? Grosberg (1988) has discussed the evolution and ecological significance of allorecognition systems in clonal invertebrate-organisms that have numerous opportunities for tissue contacts between isogeneic and allogeneic individuals. In solitary invertebrates, however, conspecific interactions rarely occur during the life cycle. Accordingly, we cannot assume that allorecognition specificity is the only phenotypic effect of genes controlling allorecognition, particularly in solitary invertebrates (Grosberg, 1988, 1989; Grosberg and Quinn, 1988). This study indicates that *L. abratsbo*, a solitary sponge, has few opportunities for tissue contacts in nature. Thus, allorecognition specificity may be an epiphenomenon resulting from pleiotropic genes. Although the ecological significance of allorecognition specificity is as yet unknown in invertebrates, pleiotropic models have been proposed and supported experimentally; *e.g.*, the control of gametic incompatibility (Oka, 1970; Scofield *et al.*, 1982; Fuke, 1983), and the discrimination of food bacteria (Wilkinson, 1984; Wilkinson *et al.*, 1984). In conclusion, this study supports the idea that self and non-self recognition is a general phenomenon in the lowest metazoan phylum, the sponges.

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Ontogenetic Variation in Sponge Histocompatibility Responses

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Abstract. Grafting of adult sponge fragments (*Chalinula* sp.) led to isograft fusion and allograft nonfusion in both parabiotic and implant grafts. We conclude that adult *Chalinula* sp. individuals discriminate between self and nonself, and fuse only isogeneic fragments. In the laboratory, however, larvae and early juveniles fuse. Larvae used in the experiments were probably genetically different, even if they were asexually reproduced. These results indicate that the capacity for fusion between allogeneic individuals disappears during ontogenesis in this sponge. In some cases, multichimeras were formed when up to five larvae fused to yield a single sponge. All 37 chimeras metamorphosed and survived during 17 days of observation. Possible mechanisms for the formation of sponge chimeras during early development are discussed, as are the costs and benefits of chimera formation at juvenile *versus* adult stages. We propose that, if fusion exists in the field, it occurs between kin larvae.

Introduction

Sessile marine organisms frequently contact each other. In many instances this contact induces a recognition process during which self/nonself histocompatibility is established, resulting in acceptance or nonacceptance of the tissues involved. Intraspecific (allogeneic) encounters are frequently characterized by visible recognition events in which various responses may occur. Allogeneic histoincompatibility (nonacceptance of tissues from different conspecific individuals) has been observed in various groups of invertebrates during the past two decades: ascidians, bryozoans, stony corals, sea anemones, gorgonians, hydrozoans, and sponges (reviewed in Grosberg, 1988). The pioneering work of Wilson (1907) led to extensive research on cellular events during the reaggregation of sponge cells (reviewed in Smith, 1988; and Gramzow *et al.*, 1989), and on the consequences of sponge grafting (*e.g.*, Hildemann *et al.*, 1979; Curtis *et al.*, 1982; Smith and Hildemann, 1986).

The general view is that, even if all sponges cannot be claimed to manifest allorecognition, increasing evidence suggests that many of them are characterized by a high degree of polymorphism, and usually will not accept allografts (Smith, 1988; Van de Vyver, 1988). These results demonstrated highly diverse reactions among sponges, to both allografts (contact between individuals of the same species) and xenografts (contact between individual of different species). The reactions vary from xenograft (Paris, 1961) and allograft acceptance (formation of stable chimeras) in various sponges (Evans and Curtis, 1979; Kaye and Ortiz, 1981; Zea and Humphreys, 1985), to allograft rejection (Smith, 1988).

The genetics of sponge allorecognition, currently, is poorly known, but can be explored through allograft experiments on transitivity relationships. Transitive compatibility is defined as a situation in which individual A is compatible with B, B is compatible with C, and A is compatible with C. A non-transitive situation occurs when A is compatible with B and C, but B and C are not compatible with each other. Allograft studies of marine sponges have demonstrated transitivity (Neigel and Avise 1983, 1985; Wulff, 1986), and may imply that complete allotypic matching is required for compatibility. A difficulty in such studies among sponge populations in the field is the possible existence of clonemates derived through asexual propagation (*e.g.*, Neigel and Avise 1983; Wulff, 1986). Therefore, the tested sponges must be widely separated from each other in the field (more than the dispersal distance for an asexual propagule), to reduce the possibility of their being clonemates.

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The phenomenon of fusion between sponge larvae has been sporadically reported (Wilson, 1907; Burton, 1949; Warburton, 1958; Borojevic, 1967; Van de Vyver, 1970; Fry, 1971; Van de Vyver and Willenz, 1975), but has received inadequate attention. Fusion between larvae derived from the same parent might be considered as an autograft if the larvae were produced parthenogenetically, as is known in corals (Stoddart, 1983, 1984) and has been suggested for some sponges (reviewed by Fell, 1974; Bergquist, 1978; Simpson, 1984). On the other hand, if the larvae are not clonemates, then their fusion may be regarded as allograft fusion, resulting in the creation of a chimera. If adult sponges do not fuse, but their allogeneic larvae fuse, then questions of variable self/nonself histo-recognition responses during a sponge's lifetime, and the capacity of larvae to distinguish between self and nonself, are raised.

In the present study, we address the following questions. How do *Chalinula* sp. adults react toward isografts and allografts? How do *Chalinula* sp. larvae derived from the same parent react to each other? And what are the consequences of encounters between larvae that originated from different parents?

Materials and Methods

We studied the brooding sponge *Chalinula* sp. from the coral reefs of Eilat, Israel, on the Red Sea (29°30′N; 34°55′E), after establishing its reproduction and settlement (Ilan and Loya, 1990). Larvae were obtained by slicing adult sponges and collecting the well-developed free-swimming larvae.

Two sets of experiments on larvae were conducted. In the first set, larvae were derived from the same parent, and in the second, from different parents. In the first set of experiments, 224 larvae were obtained from 25 individual sponges. Two to ten larvae were placed in each petri dish (all derived from the same parent) to assess the possibility of fusion between two larvae (bichimera) or more (multichimera). In the second set, 3 experiments were conducted with 104 larvae taken from 14 different individuals. Every petri dish contained only two larvae, each derived from a different adult sponge. The petri dishes (bottom surface area 9.6 cm^2) were filled with 9 ml unfiltered seawater. The adult sponges used in the second set of experiments grew in the sea, 10 to 300 m apart from each other. Such distances have been considered beyond fragment dispersal in cases of frequently fragmenting sponges growing in areas affected by storms (Jokiel *et al.*, 1982; Kaye and Ortiz, 1981; Wulff, 1985). Because fragmentation and budding are not common phenomena in *Chalinula* sp., and no frequent storms occur in the study area, such a distance between the parental sponges, diminishes the possibility that these sponges could be genetically identical clonemates. The experiments in this second set were designed to introduce larvae of every sponge to larvae from each of the other sponges. The larvae for the 3 experiments in this set were obtained from 6, 4, and 4 parental sponges and had 15, 6, and 6 possible combinations of parents, respectively (25 out of the 27 possible combination were performed in duplicate). All experiments were conducted at ambient seawater temperature (25 ± 1°C).

The tendency of larvae to aggregate was tested in the second set, using the statistical analysis of the goodness of fit (Sokal and Rohlf, 1969). According to our definition, aggregation occurs when two larvae establish and remain in contact. To calculate this occurrence, a hypothetical *Chalinula* sp. larva was considered to be a rectangle of 1×0.5 mm (0.5 mm^2). These two figures are larger than those of any *Chalinula* sp. larva measured (Ilan and Loya, 1990) and are therefore considered to be conservative. In a petri dish of 960 mm^2 bottom area, there are 1920 rectangles of 0.5 mm^2. The second larva will contact the first one only if it settles on top of the first larva, or in one of its four neighboring rectangles. Given a random larval settlement in a dish, the probability of two larvae contacting is: $5 \times (1/1920)$. Because 52 pairs of larvae were used in these experiments, there would be random contact between the larvae in $52 \times 5 \times (1/1920) = 0.135$ of the pairs. Any significantly higher value than this prediction, implies larval aggregation.

Two grafting experiments between fragments of adult sponges, involving two different protocols, were conducted *in situ* in front of the Marine Biological Laboratory, Eilat, at least 1 m below lowest tide. *Chalinula* sp. fragments of about 3×4 cm were attached to each other and to a fiberglass net anchored to the bottom. Fragments were taken from sponges situated 10 to 300 m apart from each other on the coral reef. We used five sponges in each of the two experiments, with all cross combinations (with duplicates) of allogeneic interaction made. To determine whether this species is capable of fragment fusion, all the experiment sponges were also isogeneically grafted. In the first experiment, intact external surfaces of sponges (pinacoderms) were placed in contact (parabiotic grafts). Neigel and Avise (1985) considered this technique to be more reliable than implant grafts in which a block of donor's tissue is implanted into a recipient. However, following the suggestion of Johnston and Hildemann (1982) that a reaction may be very slow, and a review by Smith (1988) on the involvement of mesohyle (inner nonfiltering) cells in the process of acceptance or rejection of grafts, we set up a second experiment in which contacts were made between fragments of mesohyle to speed the reaction process. We used equal sized fragments, and not the implant technique, to avoid a possible effect of recipient size on the donor's block (Hildemann *et al.*, 1980). The grafting experiments were observed for three months.

Samples of grafted zones on the sponges were fixed in 2.5% glutaraldehyde buffered in seawater for scanning electron microscopy, then washed, dehydrated in graded ethanol series, critical-point-dried, coated with gold-palladium and viewed in a JEOL JSM-840A SEM.

Results

When free-swimming *Chalinula* sp. larvae (n = 224), derived from the same parent sponges, were put together in a dish, 44% fused (Table I). In dishes with more than two larvae, there were cases of fusion between two larvae (bichimera as in Fig. 1a); 3 to 5 larvae (multichimera) also fused, metamorphosed successfully, and gave rise to single sponges (Fig. 1b). In all, 37 chimeras were observed (Table I). *Chalinula* sp. larvae fused during different stages: as free swimming larvae (Fig. 1a, 1b), or as post larvae shortly after attachment and metamorphosis, when they grew toward each other and fused at the contact zone (Fig. 1c). In some cases, free-swimming larvae settled on, and fused with, recently metamorphosed sponges.

In the second set of experiments, each of the pair of larvae in a dish was taken from a different sponge. In 19 pairs out of 52 (36.5%), the larvae fused. The observed number of fused pairs is significantly higher than the expected (0.135) from random settlement ($P \ll 0.001$, goodness of fit test). The larvae settled in all areas of the dishes and were not confined to certain microhabitats (*e.g.*, corners, center); therefore the aggregation was not due to external pressures. In two cases, chimeras redivided into two distinct individuals, after one to three days, although the duplicate of one of these pairs, which also produced a chimera, did not separate. Larvae and chimeras remained alive during the 17 days of observation in the first larval experiment, and in the second, they remained alive for 39 days of observation.

Isografts conducted between fragments of adult *Chalinula* sp., fused within three to ten days, whether the contact zone was between exopinacoderms (parabiotic grafts) or between mesohyles (Fig. 2). Fusion was characterized by a continuum of the choanosome, with no apparent boundary at the grafted zone. These fragments remained fused over 3 months of observation. When allografting was performed between fragments taken from the same sponges that had been used for the isografting, no fusion was observed between the 20 allografts (Fig. 3). Scanning electron micrographs of allografts attached at the internal (choanosomal) zone of the fragments, revealed that within 3 d, a gap of about 100 μm was formed between the fragments, with spicules erected toward this zone (Fig. 3b, 3c). Each fragment developed a pinacoderm at the grafted area, with a separation between them (Fig. 3d). No aggressive interactions were observed between the nonfusing fragments in the allografts. When parabiotic grafts were employed (20 pairs), both fragments remained intact, but fusion did not occur, nor was any rejection phenomenon observed over the three months of the experiment.

Discussion

The existence of self/nonself recognition among adult *Chalinula* sp. is strongly indicated in this study. Fusion between all fragments involved in isografts occurred, regardless of the grafting method used (parabiotic *versus* implant grafts), establishing that members of this species can fuse isogeneically. However, when grafts were made between allogeneic fragments of the same individuals used in isografting, fusion did not occur in any of the paired fragments.

Chalinula sp. larvae have a statistically significant tendency to aggregate. Molecules termed aggregating factors occur in some sponges (Moscona, 1968), and such molecules are known to facilitate species-specific and non-

Table I

Occurrence of fusion between larvae taken from the same Chalinula *sp. individual*

# Larvae in dish	# Dishes	% Dishes with fusion	% Fused larvae	# Chimeras of			
				2	3	4	5 Larvae
2	8	37.5	37.5	3			
3	4	50.0	41.7	1	1		
4	27	55.6	30.6	12	3	0	
5	4	75.0	65.0	0	0	2	1
6	3	33.3	11.1	1	0	0	0
7	3	100.0	71.4	2	1	2	0
9	1	100.0	66.7	0	2	0	0
10	2	100.0	95.0	0	5	1	0
Total	52	57.8	44.2	19	12	5	1

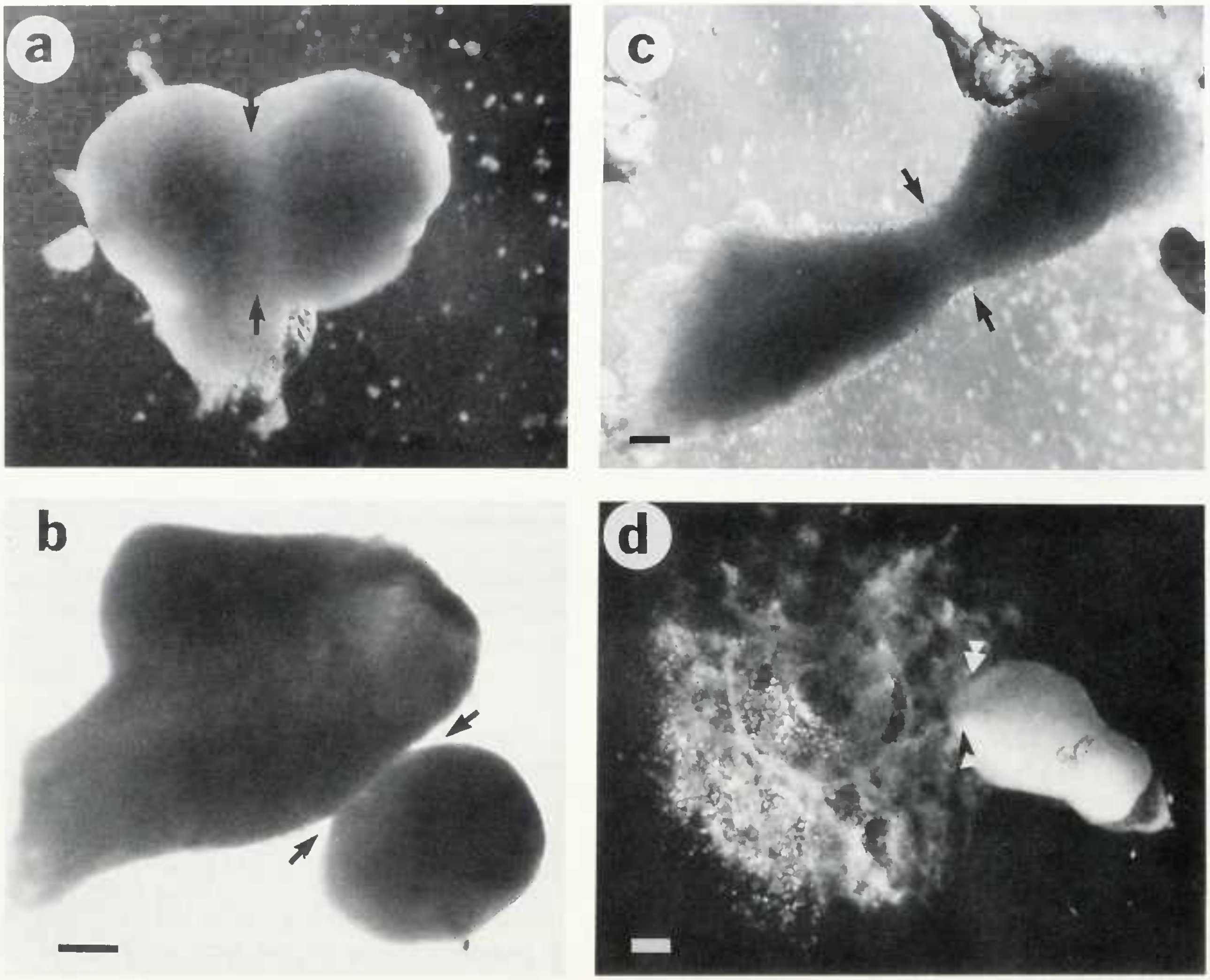

Figure 1. *Chalinula* sp. larval and post-larval fusion. (a) A pair of fused larvae, 2 h after initial contact. (b) A pair of fused larvae, 24 h after fusion, start to fuse with a third larva. (c) Fusion of two post-larvae. Fusion followed their settlement in proximity. (d) A new larva starts to settle on and fuse with a two-day old post-larva. In all the light micrographs, arrows indicate the contact zone (scale bar = 200 μm).

specific reaggregation of dissociated sponge cells (reviewed by Muller, 1982; Coombe and Parish, 1988).

The fusion of larvae derived from the same parent may have occurred for several reasons: (1) the larvae may lack a capacity for self/nonself discrimination; (2) they may possess such discrimination, but may also express an inhibition of the rejection mechanism; and (3) the larvae may have been genetically identical (products of parthenogenetic reproduction), thus resulting in the fusion of grafts that were actually isografts and not allografts.

The last cause of larval fusion is less likely, because the larvae were taken from sponges 10 to 300 m apart from each other in their natural habitat; therefore they were probably genetically different. Thus, the larvae that fused in the experiments were probably genetically different, even if asexual development of larvae occurs in *Chalinula* sp., which is unlikely (Ilan and Loya, 1990). These results differ from the situation reported by Van de Vyver and Willenz (1975), who studied the freshwater sponge *Ephydatia fluviatilis* and described larval fusion as occurring only between larvae belonging to the same strain.

If indeed larvae were incapable of self/nonself discrimination, the results with adult grafting indicate acquisition of this capability during ontogenesis. Juvenile immunological incompetence is well known among vertebrates (Cooper, 1976) and has been suggested also for corals (*e.g.*, Duerden, 1902; Lang, 1971; Hidaka, 1985) and hydroids (Teissier, 1929; Schijfsma, 1939). The tendency of *Chal-*

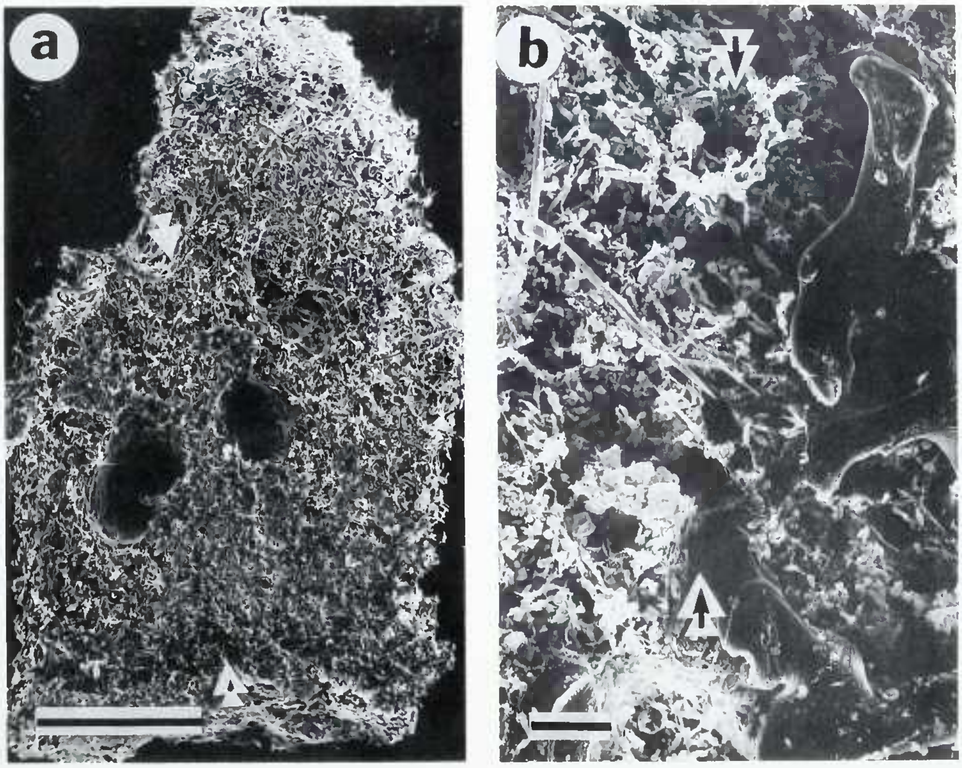

Figure 2. *Chalinula* sp. adult isograft viewed through scanning electron microscope. (a) Two complete grafted fragments, with arrows indicating toward the fusion at the contact zone (scale bar = 1 mm). (b) Higher magnification of the contact zone shows a continuum of cells (scale bar = 100 μm).

inula sp. larvae to form intraspecific aggregates demonstrates, however, some recognition capacity (though only on the species level). Lack of reaction against nonself might have been due to a lag period after which a rejection, separation, or resorption of one partner in the chimera by the other could have occurred, as is known for tunicates (Scofield *et al.,* 1982; Rinkevich and Weissman, 1987, 1989). The present study indicates that if any lag period exists, it must be at least 17–39 days long. However, a process that cannot be excluded is cell lineage competition (Buss, 1982), in which cells with different genotypes within one body may compete for position in the germ line. Finally, another option is that, although capable of differentiating self from nonself, larvae were unable to inhibit fusion due to lack or inactivation of a rejection mechanism, a situation analogous to self-tolerance in vertebrate T or B-cells (*e.g.*, Basten, 1989; Nossal, 1989; Schwartz, 1989).

Conspecific larval aggregation by *Chalinula* sp., followed by fusion with no rejection raises the question: what are the benefits of creating chimeras during larval or early post-larval stages, in contrast to the possible disadvantages (reflected by allograft incompatibility) of having chimeras at the adult stage? In the juvenile stage, the most important advantage may be the chimera size, which is larger than any of the individuals that created it. Small body size in marine invertebrates is often accompanied by high mortality, whereas larger size results in higher survivorship (*e.g.*, Loya, 1976; Ayling, 1980; Hughes and Connell, 1987). Hence, individuals that fuse, forming a chimera, immediately increase their total size and probably also their survivorship. Another possible benefit suggested for chimeras is early reproduction, because sexual maturity is also often size-related (reviewed in Harvell and Grosberg, 1988). Thus, reducing generation length may yield an increasing number of offspring per unit time, compared with a similar genotype having a longer generation time. Buss (1982) argued that chimeras might be advantageous if there is mixing of cells from all partner genotypes. Chimeras, being larger are more likely to suffer partial- rather than whole-colony mortality, with surviving cells bearing all genotypes. Finally, having a compound genotype, a chimera may gain more physiological resistance to different environmental conditions than any of its members separately (Buss, 1982; Grosberg and Quinn, 1986).

Most of the proposed benefits (except for physiological resistance) are consequences of larger body size of a chimera *versus* its members. Therefore, adults, which have already reached a substantial size, do not need to fuse with others to raise their survivorship or to reduce the

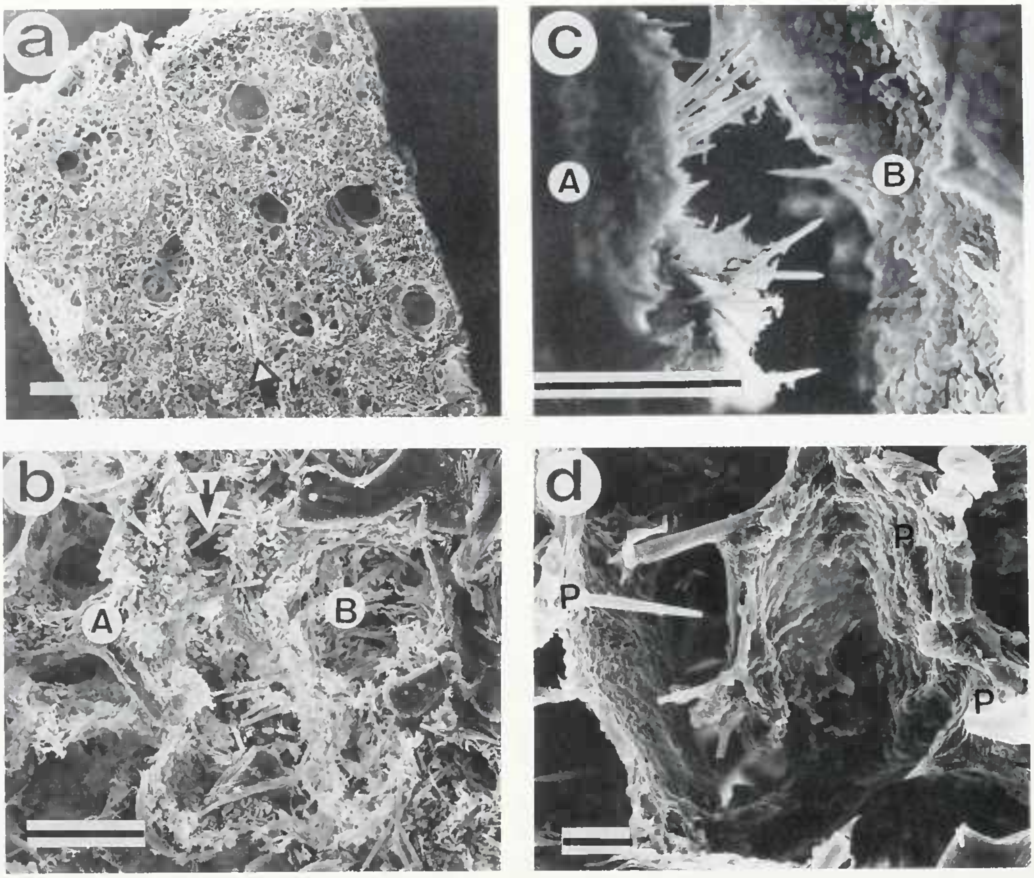

Figure 3. *Chalinula* sp. adult allografts, made by placing together the mesohyl of two fragments from different individuals and viewed through a scanning electron microscope. (a) Two allogeneic fragments. Arrow indicates the grafted area (scale bar = 1 mm). (b) Fragments A and B with a gap at the contact zone (scale bar = 100 μm). (c) Higher magnification, reveals spicules erected toward the contact zone, presumably due to cell disappearance from this area (scale bar = 100 μm). (d) Formation of a pinacoderm (P) layer by each fragment at the contact zone (scale bar = 30 μm).

time to onset of reproduction, which they have already started. We suggest, therefore, that the nonfusion of adult *Chalinula* sp. evolved because the disadvantages and risks involved are not outweighed by the chimeric benefits of larger total size.

Considering the costs of participating in a chimera, we first assume that an organism acts to maintain its integrity, in order to pass on its genotype to the next generation. Several potential deleterious consequences of creating chimeras were proposed in the literature. Buss (1982, 1983) suggested a possible parasitism by one member of a chimera on the other; by differentiating its germ cells to gametes, it would take advantage of the other member's investment in somatic tissues for maintenance. Other workers have demonstrated oriented translocation of material in coral chimeras (Rinkevich and Loya, 1983), possible transmission of pathogens (Buss, 1982), or in an ascidian, total resorption of one member's soma by its partner, under laboratory mariculture (Rinkevich and Weissman, 1987, 1989). In this study, although all the chimeras survived at least 17 days, the fate of the cells of each partner was not determined.

Chalinula sp. larval fusion has been observed experimentally in this study in the laboratory. However, its frequency in nature is unknown. Theoretically, the chances of contact between *Chalinula* sp. larvae from different sources in the field are small. Its year-round reproductive pattern (Ilan and Loya, 1990) leads to a small number of free-swimming larvae in the population at any given time. This fact, together with the large distance between adult colonies, relative to larval size, plus rapid larval settlement (1 to 8 h after release) (Ilan and Loya, 1990), contributes to the low probability of larval contact. Nonetheless, larvae brooded in the same sponge, even if they are genetically different (produced sexually), may overcome most of the barriers to larval fusion in the field. Such larvae are in close proximity and, if spawned synchronously, may settle together and fuse. Fusion between kin larvae may provide an additional selective advantage. Kin larvae partially share genotypes, therefore the survival of each is a partial success for the other genotype. Thus, if larval and newly post-larval chimeras of *Chalinula* sp. do occur in the field, we assume they will be primarily among kin.

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Relationship Between Body Size, Growth Rate, and Maximal Enzyme Activities in the Brine Shrimp, *Artemia franciscana*

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Abstract. Activity-body size relationships for eight enzymes (*citrate synthase*, CS; *lactate dehydrogenase*, LDH; *pyruvate kinase*, PK; *alanine aminotransferase*, ala AT; *aspartate aminotransferase*, asp AT; *glutamate dehydrogenase*, GDH; *glucose-6-phosphate dehydrogenase*, G6Pdh; and *nucleoside diphosphate kinase*, NDPK) were examined in the brine shrimp, *Artemia franciscana*. The animals were fed on the alga *Dunaliella salina*, which was provided in three concentrations representing a 25-fold range. Enzyme activities per animal (Y) were regressed against body size (M, expressed as dry mass or protein) in the form of the allometric equation, $\log Y = \log a + b \log M$, where a and b are fitted constants. For all enzymes considered, the value of the scaling exponent (b) was significantly higher when dry mass was used, as a body size index, than when protein mass was used. Therefore, the index of body size chosen can influence the exponent obtained in allometric studies. Although specific growth rates of different cultures varied greatly, no significant differences in scaling relationships were found between cultures for any enzyme. For many enzymes, growth rate may not be a source of variation in scaling relationships. Unlike the other enzymes examined, the log-transformed NDPK activity *versus* log-transformed mass was not linear; NDPK activity reached a plateau. Variation in NDPK scaling relationships with growth may provide a means to predict growth rate in *Artemia*.

Introduction

Relationships between metabolic rate processes and body size are usually described by an allometric equation of the form:

$$Y = aM^b \qquad (1)$$

where Y is the rate process, M is body mass and a and b are empirically derived constants (*e.g.*, Brody, 1945; Kleiber, 1961; Peters, 1983). A much-disputed feature of this equation is the precise value of the exponent b (here referred to as the "scaling exponent"). For interspecific studies of respiration in mammals, for example, the exponent 0.75 is often cited (Peters, 1983), yet arguments have been made that the true exponent is 0.67, based on dimensional analyses (*e.g.*, Mahon and Bonner, 1983) or statistical considerations (*e.g.*, Heusner, 1982).

Disagreements such as these are difficult to resolve for a number of reasons. The measurement of a rate variable, such as respiration, has several potential sources of variation including the organism's nutritional status and activity level (Peters, 1983). The result is that data in the literature relating metabolic rate and body size may not be directly comparable. Careful analyses of the sources of variation in scaling relationships are needed to overcome these problems.

At least two sources of variation have not been accounted for in previous scaling studies: the index of body size used and the growth rate. The index of body size used may be an important source of variation, particularly in intraspecific studies where different developmental stages are compared. In mammals, for example, body composition varies widely as they mature; bone and other structural tissues comprise a larger portion of the mass of young mammals, while lipid stores increase disproportionately with size and age (Taylor, 1980). In invertebrates, body composition is much more variable, even within adult organisms, and it is also affected by food concentration (Mayzaud, 1986). The variable traditionally selected for use in scaling studies is fresh mass. Because a large portion of fresh mass is water, substantial error can be introduced due to dehydration or, in the case of aquatic organisms, water associated with the outer surface. As an alternative, dry mass is more reproducible, particularly for small or-

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ganisms (Downing and Rigler, 1984), but comparisons of dry mass imply, often unjustifiably, a constancy of composition. Ideally, what is required is a mass variable highly correlated with the metabolically active mass of tissue (because this is most relevant to a metabolic rate variable). Protein constitutes a relatively constant proportion of mass across developmental stages in mammals (Brody, 1945; Kleiber, 1961; Schmidt-Nielsen, 1984), and may also meet the requirements for a mass variable in invertebrates.

Growth has been defined by Bertalanffy (1957) as the difference between catabolic and anabolic processes. Bertalanffy suggested that these processes vary with body size in different ways. If this is true, then relationships between integrative measures of metabolism (*i.e.*, respiration) and body size may change with growth rate of the organisms. For example, comparing the respiration-body size relationships of a fast-growing group of animals in which anabolic processes dominate, with a more slowly growing group in which catabolism and anabolism are more nearly balanced, may yield conflicting results.

In this study, we address the issue of the sources of variation in scaling relationships. We ask, in particular, whether the index of body size used or the growth rate of the organism can affect the scaling exponent. Because of the wide range of parameters that can influence an index of metabolic rate, such as respiration, we examined the maximal activities of selected enzymes as alternative rate variables. Enzyme assays can be conducted under defined conditions, and afford high precision. For an experimental organism we selected the brine shrimp, *Artemia franciscana*, a crustacean whose growth rate can be manipulated easily in the laboratory. Growth in *Artemia* is exponential for the early part of its life history (Berges, Roff, and Ballantyne, unpub.), which means that, over a particular range of body size, growth rate is not correlated with body mass. This independence is a necessary precondition for distinguishing the effect of growth rate from the effects of body mass. By comparing enzyme activity-body size relationships among groups of animals growing at different rates, we could determine whether growth rate can be considered an important source of variation in scaling relationships. In addition, by measuring both protein and dry mass we could examine the effects of different mass variables on scaling relationships.

Enzymes selected for assay were as follows: *citrate synthase* (CS), a rate-limiting enzyme in the Krebs cycle that provides an index of aerobic metabolic rate (Hochachka *et al.*, 1970); *lactate dehydrogenase* (LDH), which allows an assessment of anaerobic metabolic capacity (Somero and Childress, 1980); *pyruvate kinase* (PK), which provides an indication of maximal potential flux through glycolysis; *aspartate* and *alanine aminotransferase* (ala AT and asp AT), which function in amino acid metabolism in both synthetic and degradative pathways; *glutamate dehydrogenase* (GDH), which may provide an index of nitrogen excretion and, therefore, of the potential flux toward amino acid degradation; and *glucose-6-phosphate dehydrogenase* (G6Pdh) and *nucleoside diphosphate kinase* (NDPK), two primarily anabolic enzymes whose activities might be expected to respond to growth differences. G6Pdh is the rate-limiting enzyme in the hexose monophosphate shunt (Hochachka and Somero, 1984), providing both NADPH, the primary reducing power for anabolic processes, and ribulose sub-units for nucleic acid synthesis. NDPK acts to transfer energy from ATP into the other triphosphate nucleotides (*e.g.*, CTP, UTP, ITP) that are used preferentially in synthesis pathways; it may therefore control the allocation of energy to growth processes.

Materials and Methods

Cysts of *Artemia franciscana* SFB were obtained from San Francisco Bay Brand Inc. (Newark, California); all animals used were hatched from a single lot. Brine shrimp were hatched in 18 ‰ seawater (Instant Ocean Brand, Aquarium Systems, Mentor, Ohio) at 25°C in a 60-l common aquarium. Approximately 1000 animals were transferred to each of three 20-l aquaria for the feeding experiments. The green alga, *Dunaliella salina*, which is a suitable food item for *Artemia* (Mason, 1963; Reeve, 1963), was grown in 35 ‰ seawater in f/2 medium (Guillard and Ryther, 1962).

On two separate occasions (trials 1 and 2), three food levels were established to provide a range of growth conditions based on growth results obtained by Reeve (1963). These were: 2000 cells $\cdot$ ml^{-1} (low food, L), 10,000 cells $\cdot$ ml^{-1} (medium food, M) and 50,000 cells $\cdot$ ml^{-1} (high food, H). Shrimp were placed in aquaria with food soon after hatch. The batch culture was sieved with a 350 μm net to ensure that only hatched nauplii of uniform size were introduced to each culture. The concentration of algal cells was monitored with a light microscope and a Levy Ultraplane haemocytometer (Guillard, 1978), and the cells were replenished once daily. Each day of the experiment, 6 random samples of 25 shrimp each were taken from each culture. Three samples from each culture were analyzed for protein content and, on alternate days, for enzyme activities. The remaining three groups from each culture were preserved in 2% formalin for later dry mass measurement.

Brine shrimp were homogenized in 50–1000 μl of ice-cold 50 m*M* imidazole buffer, pH 7.4, using a Megason PA-300 sonicator at maximum setting for three bursts of 10 s each. The homogenates were of sufficiently low optical density that centrifugation was not required.

The protein level of the homogenates was found by microassay, the procedure based on the Coomassie brilliant blue assay of Bradford (1976) [Bio-Rad Laboratories (Canada) Inc., Mississauga, Ontario] with bovine serum albumin as the standard. Preliminary experiments showed that imidazole buffer did not affect the linearity of the assay.

Maximum enzyme activities were determined using a Varian DMS 100 UV-Visible spectrophotometer equipped with a thermostatted cell changer maintained at 25° (±0.1°) C with a Haake D8 circulating temperature bath. Reaction rates were determined by increase or decrease in absorbance of NADH or NADPH at 340 nm. *Citrate synthase* was monitored at 412 nm using 5,5′ dithiobis 2-nitrobenzoic acid (DTNB). Enzyme activity was expressed as units per animal, where one unit equals 1 μmol substrate converted to product per minute. Conditions for assay procedures were adapted from Hochachka *et al.* (1970) and Emmett and Hochachka (1981), unless otherwise indicated, with substrate and cofactor concentrations optimized to give maximum activity. All assays were conducted in 50 m*M* imidazole pH 7.2, except CS, which was assayed at pH 8.2. Other conditions were as follows:

Citrate synthase (CS), EC 4.1.3.7: 0.1 m*M* DTNB, 7.0 m*M* $MgCl_2$, 0.3 m*M* acetyl coenzyme A, 0.5 m*M* oxaloacetate (omitted from control).

Lactate dehydrogenase (LDH), EC 1.1.1.27: 0.2 m*M* NADH, 100 m*M* KCl (Somero and Childress, 1980), 2.0 m*M* sodium pyruvate (omitted from control).

Pyruvate kinase (PK), EC 2.7.1.40: 0.2 m*M* NADH, 5.0 m*M* ADP, 50 m*M* KCl, 10 m*M* $MgCl_2$, 5.0 m*M* phosphoenolpyruvate (omitted from control) and excess lactate dehydrogenase.

Alanine aminotransferase (ala AT), EC 2.6.1.2: 200 m*M* L-alanine, 0.2 m*M* NADH, 0.025 m*M* pyridoxal phosphate, excess LDH and 10.5 m*M* alpha-ketoglutarate (omitted from control).

Aspartate aminotransferase (asp AT), EC 2.6.1.1: 0.2 m*M* NADH, 7.0 m*M* alpha-ketoglutarate, 0.025 m*M* pyridoxal phosphate, excess malate dehydrogenase, and 30 m*M* L-aspartate (omitted from control).

Glutamate dehydrogenase (GDH), EC 1.4.1.2: 0.2 m*M* NADH, 250 m*M* ammonium acetate, 0.1 m*M* Na_2EDTA, 1.0 m*M* ADP, and 14.0 m*M* alpha-ketoglutarate (omitted from control).

Nucleoside diphosphate kinase (NDPK), EC 2.7.4.6: 0.2 m*M* NADH, 20 m*M* $MgCl_2$, 2.0 m*M* ATP, 70 m*M* KCl, 1.1 m*M* phosphoenolpyruvate, excess LDH, excess PK, and 0.7 m*M* thymidine diphosphate. Two controls were run; homogenate was omitted from one, and thymidine diphosphate from the other (Agarwal *et al.*, 1978).

Glucose - 6 - phosphate dehydrogenase (G6Pdh), EC 1.1.1.49: 0.4 m*M* NADP, 7.0 m*M* $MgCl_2$, 1.0 m*M* glucose-6-phosphate (omitted from control).

Because the cultures differed markedly in their concentrations of *Dunaliella salina*, we determined whether enzymes in the algae could bias determinations of enzymes in *Artemia*. Known volumes of culture were filtered and homogenized, as for *Artemia*, and were centrifuged at 18,000 × *g* for 10 min in a Sorvall RCB-5 refrigerated centrifuge. Enzyme assays were performed on the supernatants under conditions identical to those used for *Artemia*.

In the smallest post-yolk sac nauplii, *Dunaliella* could contribute, at most, 1.2% of asp AT activity measured in *Artemia;* this is well within the error for replicate determinations. Similar analyses for other enzymes show even less potential for interference.

The mass of preserved samples of brine shrimp was determined about 3 weeks after fixation. Animals were rinsed well in deionized water, placed in pre-massed pans, and dried for 12 h at 50°C. Immediately upon removal from the drying oven, samples were brought to room temperature in a desiccator, then allowed to equilibrate with room humidity for 30 min before final determination of mass. Room temperature was 24°C and relative humidity 50%. This procedure is preferable to the time-dependent bias in mass that results from sequential removal of samples from a common desiccator. The mass added by room humidity is about 6% and is highly reproducible between samples (Chisholm and Roff, 1990). To estimate errors due to preservation, mass was determined for freshly dried, and preserved-then-dried animals from a separate culture growing under high food conditions.

Statistical analyses and model fitting were carried out according to the GLM (general linear model) and NLIN (non-linear fitting) procedures (SAS Institute, Cary, North Carolina). A linear regression of dry mass on protein mass was performed using a functional regression; such a regression is appropriate in cases where both variables have similar associated error (Ricker, 1973). Similarly, functional regression was used to compare freshly dried animals with preserved-then-dried animals.

Changes in dry mass and protein mass over time were modeled for each culture treatment using the simple exponential growth model:

$$M_t = M_0 e^{gt} \qquad (2)$$

where M_t is mass at time t, t is expressed in days, and M_0 and g are fitted constants. The Gauss-Newton method (SAS Institute) was used for model fitting. For each enzyme, activity per whole animal was regressed against dry or protein mass for individual culture treatments and for pooled data. Tests of homogeneity of regression slopes and intercepts for culture treatments were performed in an analysis of covariance. Where found, differences were investigated using Fisher's least significant difference

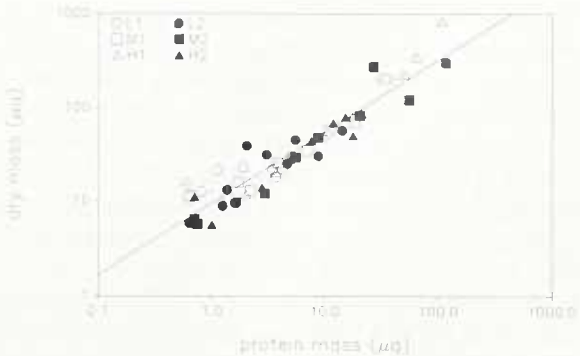

Figure 1. Dry mass *versus* protein mass for *Artemia franciscana* SFB under different culture treatments (L = 2000, M = 10,000 and H = 50,000 cells · ml^{-1} of *Dunaliella salina*). Error bars give standard errors of mean mass (n = 3). Equation of the functional regression line is: log Y = 1.663 + 0.763 log X, with r^2 = 0.893.

(LSD) test (Steel and Torrie, 1980). For all comparisons, the probability of a type I error was set at 0.05.

Results

Dry- and protein mass relationships and preservation effects

Regression analysis revealed non-normality and increasing variance with mean mass. Data (Fig. 1) fit the linear functional regression model,

$$\log(\text{dry mass in }\mu g) = 1.663 + 0.763 \log(\text{protein mass in }\mu g)$$

with r^2 of 0.893 and 22.4 percent standard error of the estimate. This relationship was used to convert protein mass to equivalent dry mass without introducing additional variation.

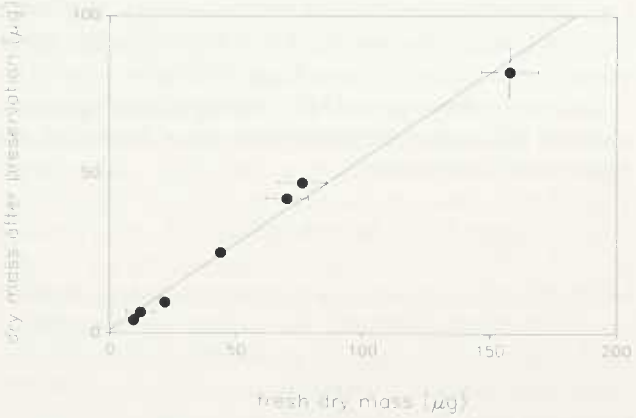

Figure 2. Dry mass after 3 weeks preservation in 2% formalin *versus* dry mass in freshly killed *Artemia franciscana* SFB in a culture fed 50,000 cells · ml^{-1} of *Dunaliella salina*. Error bars give standard errors of mean mass (n = 3). Functional regression equation is Y = 0.567 X, with r^2 = 0.985.

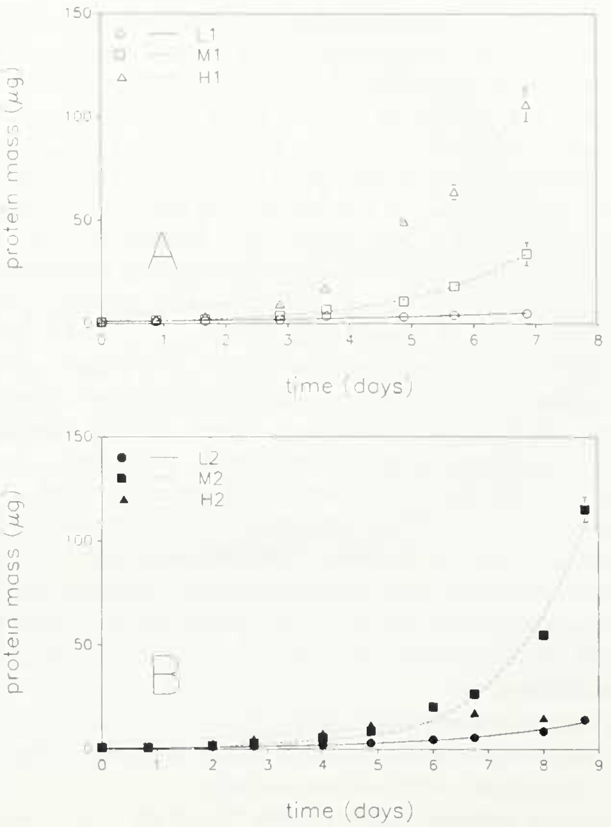

Figure 3. Protein mass growth of *Artemia franciscana* SFB under different culture treatments (L = 2000, M = 10,000 and H = 50,000 cells · ml^{-1} of *Dunaliella salina*) for trial 1 (A) and trial 2 (B). Error bars give standard errors of mean mass (n = 3 groups of 25 animals). Curves represent best fits of an exponential growth model. Model parameters are given in Table I.

For the loss-of-mass experiment, functional regression gave the relationship:

$$\text{preserved dry mass} = 0.567 \text{ fresh dry mass}$$

with r^2 of 0.985 (Fig. 2). Thus, preservation introduced a mass loss of 43.3%, consistent across all sizes of animals. This correction factor was therefore applied to all masses of preserved animals.

Culture growth rates

Identical food concentrations did not produce identical growth rates in separate trials (Fig. 3 for protein mass; Fig. 4 for dry mass). Water conditions in the high food culture in trial 2 became poor, and subdued animal swimming activity was noted on day 7. Therefore, the data obtained after day 6 were excluded from the exponential growth model for culture H2 (although points are plotted in Figs. 3 and 4). An examination of exponential

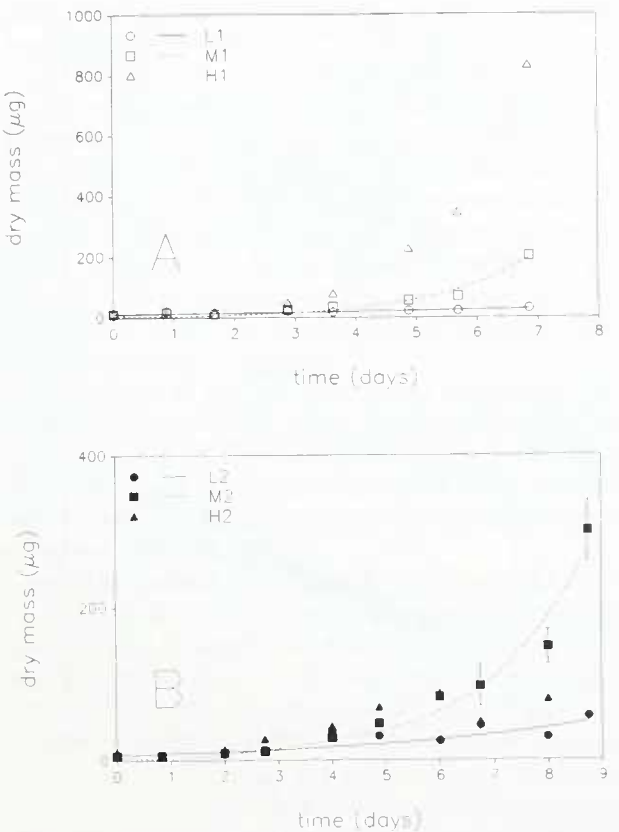

Figure 4. Dry mass growth of *Artemia franciscana* SFB under different culture treatments (L = 2000, M = 10,000 and H = 50,000 cells · ml^{-1} of *Dunaliella salina*) for trial 1 (A) and trial 2 (B). Error bars give standard errors of mean mass (n = 3 groups of 25 animals). Curves represent best fits of an exponential growth model. Model parameters are given in Table I.

growth models (Table I) demonstrates a broad range of growth rates. The parameters for the models were consistent with a more rapid increase in protein mass than in dry mass; in all cases except H2, the value of the exponent g for protein is higher than that for dry mass.

Despite differences in growth rate, a qualitative examination of preserved individuals indicated that the cultures did not have clearly different development. Compound eye and limb bud development was more advanced in individuals in the higher food cultures, but these differences could not be defined based on the stages of development in *Artemia* indicated by Heath (1924) and Anderson (1967).

Enzyme activities

For the majority of enzymes, there were no visually discernible differences between activities in animals from different cultures. This generalization held true for CS (Fig. 5A), LDH (Fig. 5B), PK (Fig. 5C), asp AT (Fig. 5D), ala AT (Fig. 5E), GDH (Fig. 5F), and G6Pdh (Fig. 5G) and even for the abnormal culture conditions for H2.

Only in the case of NDPK were differences in enzyme activity apparent between cultures. For clarity, only data from trial 1 (which exhibited the greatest range of growth rates) are presented in Figure 6A, B. At low mass, enzyme activities for different treatments overlapped, but, as animal mass increased, activities in different culture treatments became distinct. For NDPK activity, non-linearity in both log-transformed and in untransformed data was apparent, and activities reached a plateau at higher mass. This is most clearly seen in non-transformed data (Fig. 6B).

Although protein and dry mass yielded similar enzyme activity-body size relationships, slopes were greater for dry mass. Statistical analyses of regression data (Table II) confirm this. In general, while the slopes of regression of log-transformed data (scaling exponents) were greater than or equal to 1.0 for the dry mass data, for the protein mass data the exponents tended to be equal to or less than 1.0. On average, dry mass scaling exponents were 0.29 higher than those calculated for protein. Statistically significant differences were found for scaling exponents, but, intercepts (a) were not different in any case ($P > 0.07$ in all cases). In the following discussion, the term "global" refers to the scaling exponent for all data combined, while "spe-

Table I

Parameters (with asymptotic 95% confidence intervals) describing growth of Artemia franciscana *SFB under different culture treatments, using the exponential growth model** $M_t = M_0e^{gt}$

A) Protein mass

Culture	M_0 (asymptotic 95% CI)	g (asymptotic 95% CI)
L1	0.962 (0.195–1.72)	0.279 (0.077–0.482)
L2	0.560 (0.431–0.689)	0.342 (0.303–0.380)
M1	1.06 (0.52–1.60)	0.479 (0.363–0.595)
M2	0.626 (0.256–0.995)	0.560 (0.466–0.652)
H1	0.881 (0.782–0.979)	0.826 (0.802–0.850)
H2	1.050 (0.54–1.56)	0.495 (0.387–0.605)

B) Dry mass

Culture	M_0 (asymptotic 95% CI)	g (asymptotic 95% CI)
L1	12.5 (7.94–14.3)	0.110 (0.036–0.256)
L2	5.81 (3.15–9.09)	0.305 (0.180–0.429)
M1	7.84 (5.92–9.82)	0.399 (0.298–0.501)
M2	3.38 (2.74–4.04)	0.524 (0.469–0.587)
H1	5.83 (3.40–9.84)	0.783 (0.708–0.859)
H2	5.80 (3.66–7.94)	0.509 (0.363–0.656)

* M_0 is initial mass (µg), M_t is mass at time t, and t is time in days. Culture treatments are concentrations of the alga *Dunaliella salina* (L = 2000 cells · ml^{-1}, M = 10, 000 cells · ml^{-1} and H = 50,000 cells · ml^{-1}).

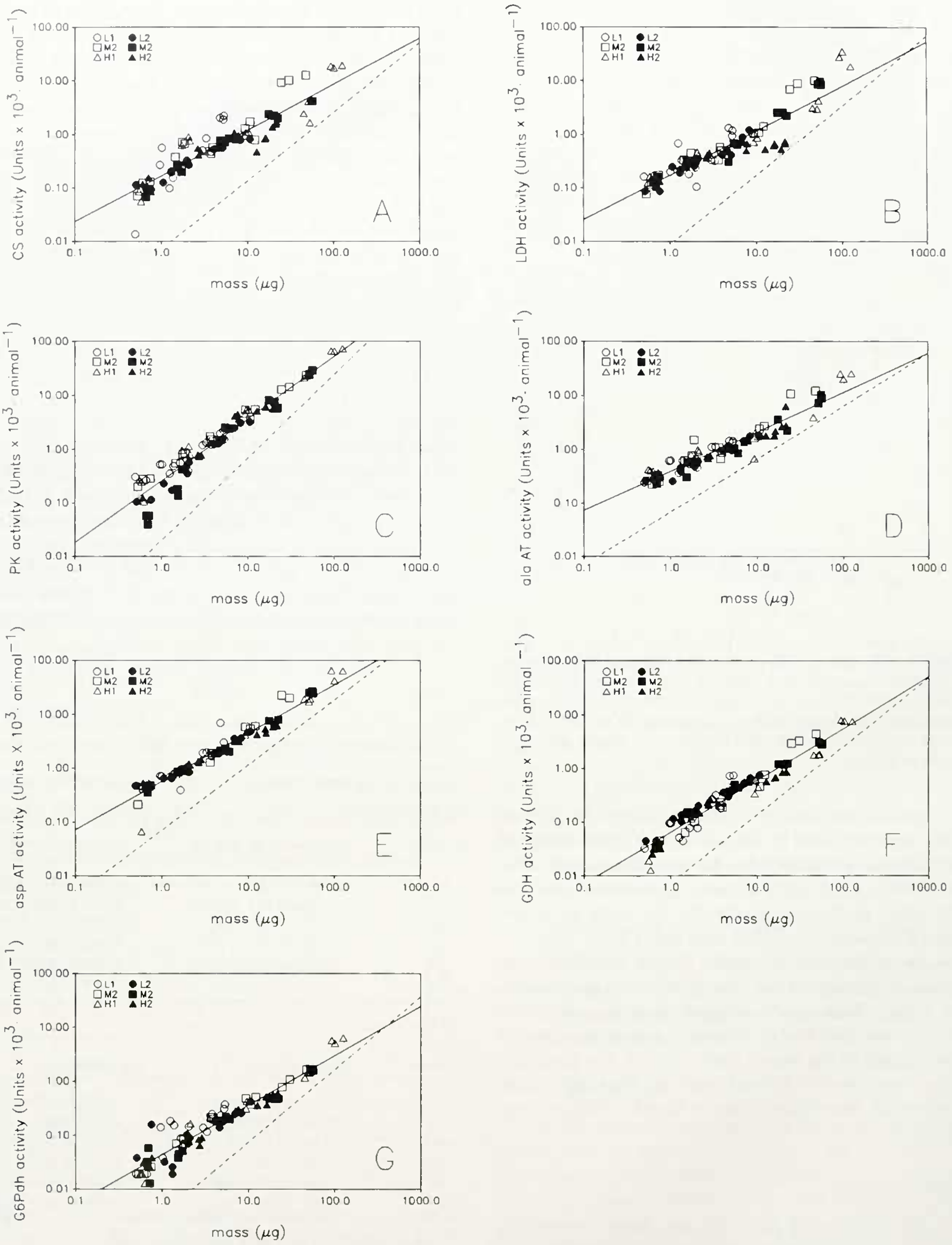

Figure 5. Log-transformed enzyme activity *versus* log-transformed dry mass and protein mass for A, citrate synthase (CS); B, lactate dehydrogenase (LDH); C, pyruvate kinase (PK); D, alanine aminotransferase (ala AT); E, aspartate aminotransferase (asp AT); F, glutamate dehydrogenase (GDH); and G, glucose-6-

cific" refers to scaling relationships in individual culture treatments (see Table II).

CS activity scaled to a global exponent of 1.13 based on dry mass. Specific exponents were generally near 1.0, except culture L1, which was significantly higher than 1.0 ($P < 0.02$). Tests revealed significantly different slopes between cultures ($P < 0.03$). LSD tests identified L1 as the sole distinct culture. In terms of protein mass, the global exponent was 0.864, significantly different from 1.0 and 0.75. As observed for dry mass data, scaling exponents of L1 and H2 cultures were significantly different from those of other cultures.

LDH scaled to a global exponent of 1.07 for dry mass data and 0.812 for protein data. Significant differences in specific scaling exponents were found for dry mass data ($P < 0.01$), and for protein data ($P < 0.01$). However, only L1 and H2 cultures were significantly different when examined with LSD tests.

PK data yielded global scaling exponents of 1.56 for dry mass, and 1.18 for protein mass, both significantly higher than 1.0. Significant differences were detected in both the dry mass ($P < 0.01$) and protein mass data ($P < 0.01$). LSD tests showed culture L1 to be distinct.

Asp AT activity scaled to a global exponent generally higher than 1.0 (1.21) for dry mass, yet lower than unity (0.94) for protein mass. Heterogeneity of scaling exponents was found in both cases: $P < 0.01$ for dry mass, $P < 0.01$ for protein, with LSD tests identifying H2 and L2 cultures as significantly different from others.

For ala AT activity, the global scaling exponent was 0.984 for dry mass (not significantly different from 1.0, $P < 0.67$), but 0.747 (significantly lower than 1.0, but not different from 0.75 ($P < 0.01$, $P < 0.91$, respectively) for protein data. In both cases, specific scaling exponents were not significantly different from one another ($P < 0.39$ for dry mass, $P < 0.39$ for protein).

GDH activity scaled to a global exponent of 1.28 for dry mass, significantly higher than 1.0 ($P < 0.01$); and 0.978 for protein data, not significantly different from 1.0 ($P < 0.45$). As was the case for CS activity, specific slope differences were detected for dry mass ($P < 0.02$) and protein mass ($P < 0.03$). LSD tests showed that only culture H2 was significantly different.

NDPK data were more variable with respect to specific scaling exponents for both protein and dry mass than for any other enzyme. The global exponent for dry mass was 1.24, significantly greater than 1.0 ($P < 0.01$). Specific exponents varied from 0.327 to 2.06 and significant differences were found ($P < 0.01$). A similar picture emerged from protein mass analysis; the global exponent of 0.942 (not significantly different from 1.0, $P < 0.35$) had a specific range of 0.189 to 1.57 (significant differences found, $P < 0.01$). LSD tests identified four groupings for both protein and dry mass data: L1 and L2 were distinct, while H1 and M1 fell together, and H2 and M2 formed another grouping.

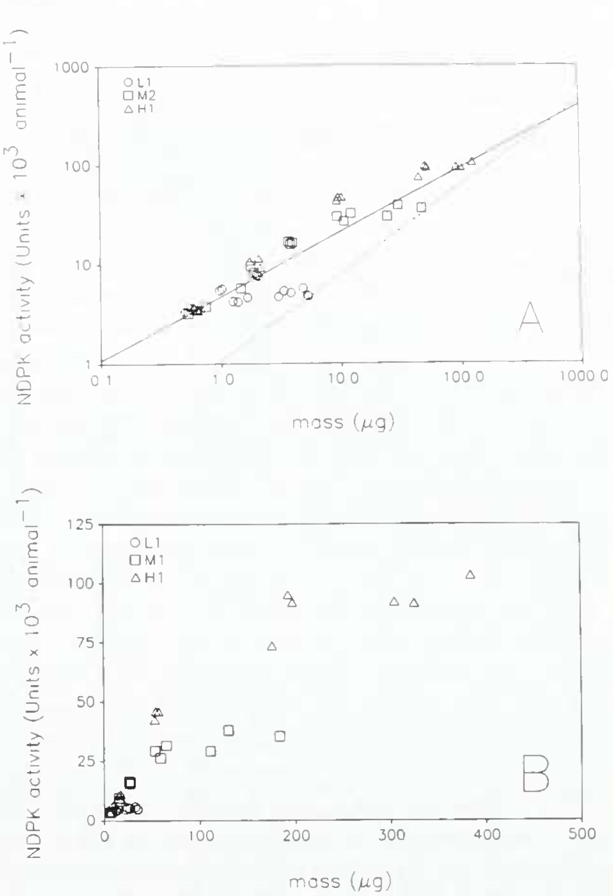

Figure 6. A, Log-transformed nucleoside diphosphate kinase (NDPK) activity versus log-transformed dry mass and protein mass in *Artemia franciscana* SFB under different culture treatments (L = 2000, M = 10,000 and H = 50,000 cells · ml^{-1} of *Dunaliella salina*) for trial 1 data. Solid line represents protein mass regression, dashed line represents dry mass regression. Data points are presented only for protein mass data. Linear regression parameters are given in Table II. B, Linear plot of the same data, dry mass only.

For G6Pdh, activity scaled to a global exponent of 1.26 for dry mass (significantly greater than 1.0, $P < 0.01$) and 0.961 for protein mass (not significantly different from 1.0, $P > 0.21$). The specific scaling exponents were not

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phosphate dehydrogenase (G6Pdh) in *Artemia franciscana* SFB under different culture treatments (L = 2000, M = 10,000 and H = 50,000 cells · ml^{-1} of *Dunaliella salina*). Solid line represents protein mass regression, dashed line represents dry mass regression. Data points are presented only for protein mass data. Linear regression parameters are given in Table II.

Table II

Parameters (±S.E.M.) for regression of enzyme activity (Units · animal^{-1}) versus *mass (μg) for* Artemia franciscana *SFB under different culture treatments*

		Protein mass				Dry mass					
				H_0 test				H_0 test			
Enzyme	Culture	log a	b	b = 0.75	b = 1.00	log a	b	b = 0.75	b = 1.00	r^2	%SEE
CS	ALL	−0.78 ± 0.04	0.86 ± 0.05	0.02	0.01	−1.61 ± 0.08	1.13 ± 0.06	0.01	0.02	0.83	75.0
	H1	−0.71 ± 0.13	0.85 ± 0.10	0.34	0.16	−1.53 ± 0.21	1.12 ± 0.13	0.01	0.39	0.84	101.6
	M1	−0.65 ± 0.13	0.86 ± 0.17	0.54	0.43	−1.48 ± 0.28	1.13 ± 0.23	0.13	0.59	0.73	78.4
	L1	−0.96 ± 0.11	1.71 ± 0.27	0.01	0.03	−2.62 ± 0.34	2.24 ± 0.36	0.01	0.01	0.80	94.3
	H2	−0.67 ± 0.07	0.61 ± 0.08	0.09	0.01	−1.26 ± 0.14	0.80 ± 0.10	0.64	0.07	0.84	39.7
	M2	−0.84 ± 0.04	0.88 ± 0.05	0.02	0.04	−1.70 ± 0.08	1.16 ± 0.06	0.01	0.02	0.97	27.2
	L2	−0.81 ± 0.02	0.82 ± 0.05	0.19	0.01	−1.60 ± 0.07	1.07 ± 0.06	0.01	0.27	0.97	16.1
LDH	ALL	−0.76 ± 0.03	0.81 ± 0.04	0.16	0.01	−1.56 ± 0.08	1.07 ± 0.06	0.01	0.21	0.83	71.5
	H1	−0.74 ± 0.11	0.91 ± 0.09	0.09	0.30	−1.61 ± 0.18	1.20 ± 0.11	0.01	0.11	0.90	81.6
	M1	−0.73 ± 0.09	0.87 ± 0.12	0.32	0.31	−1.58 ± 0.19	1.16 ± 0.16	0.03	0.34	0.86	48.9
	L1	−0.67 ± 0.10	0.66 ± 0.25	0.73	0.21	−1.21 ± 0.31	0.73 ± 0.39	0.97	0.51	0.28	90.3
	H2	−0.68 ± 0.06	0.43 ± 0.06	0.01	0.01	−1.12 ± 0.12	0.58 ± 0.09	0.07	0.01	0.79	32.5
	M2	−0.78 ± 0.07	0.91 ± 0.07	0.04	0.21	−1.67 ± 0.12	1.20 ± 0.08	0.01	0.04	0.95	11.9
	L2	−0.77 ± 0.03	0.79 ± 0.07	0.36	0.02	−1.56 ± 0.09	1.06 ± 0.09	0.01	0.56	0.92	24.3
PK	ALL	−0.59 ± 0.03	1.18 ± 0.04	0.01	0.01	−1.74 ± 0.06	1.56 ± 0.05	0.01	0.01	0.93	58.0
	H1	−0.33 ± 0.03	1.04 ± 0.02	0.01	0.10	−1.34 ± 0.05	1.36 ± 0.03	0.01	0.01	0.99	16.7
	M1	−0.40 ± 0.04	1.05 ± 0.05	0.01	0.33	−1.43 ± 0.08	1.39 ± 0.06	0.01	0.02	0.98	18.3
	L1	−0.40 ± 0.06	0.92 ± 0.12	0.21	0.47	−1.24 ± 0.17	1.13 ± 0.18	0.06	0.47	0.81	34.4
	H2	−0.62 ± 0.07	1.17 ± 0.08	0.01	0.04	−1.77 ± 0.14	1.55 ± 0.09	0.01	0.01	0.95	39.1
	M2	−0.92 ± 0.08	1.41 ± 0.08	0.01	0.01	−2.33 ± 0.15	1.87 ± 0.11	0.01	0.01	0.97	55.1
	L2	−0.80 ± 0.05	1.40 ± 0.09	0.01	0.01	−2.20 ± 0.13	1.87 ± 0.13	0.01	0.01	0.95	34.9
ala AT	ALL	−0.42 ± 0.03	0.75 ± 0.03	0.91	0.01	−1.16 ± 0.05	0.98 ± 0.04	0.01	0.67	0.90	47.4
	H1	−0.33 ± 0.10	0.73 ± 0.08	0.80	0.01	−1.04 ± 0.16	0.96 ± 0.09	0.05	0.70	0.89	68.9
	M1	−0.40 ± 0.10	0.82 ± 0.14	0.59	0.23	−1.21 ± 0.22	1.09 ± 0.18	0.09	0.64	0.80	57.7
	L1	−0.40 ± 0.04	0.78 ± 0.10	0.77	0.04	−1.18 ± 0.14	1.05 ± 0.15	0.08	0.73	0.84	28.2
	H2	−0.40 ± 0.05	0.66 ± 0.06	0.13	0.01	−1.05 ± 0.11	0.87 ± 0.07	0.13	0.11	0.92	28.1
	M2	−0.47 ± 0.06	0.77 ± 0.06	0.89	0.01	−1.22 ± 0.11	1.00 ± 0.08	0.06	0.97	0.94	37.6
	L2	−0.45 ± 0.03	0.70 ± 0.05	0.39	0.01	−1.14 ± 0.07	0.93 ± 0.07	0.02	0.29	0.95	17.0
asp AT	ALL	−0.25 ± 0.02	0.92 ± 0.03	0.01	0.01	−1.15 ± 0.05	1.21 ± 0.04	0.01	0.01	0.94	41.1
	H1	−0.37 ± 0.09	1.03 ± 0.07	0.01	0.72	−1.38 ± 0.15	1.36 ± 0.09	0.01	0.01	0.95	65.3
	M1	−0.31 ± 0.05	1.07 ± 0.07	0.01	0.36	−1.34 ± 0.12	1.40 ± 0.09	0.01	0.01	0.96	26.7
	L1	−0.21 ± 0.07	1.02 ± 0.18	0.16	0.91	−1.23 ± 0.26	1.37 ± 0.28	0.05	0.22	0.72	59.3
	H2	−0.15 ± 0.02	0.71 ± 0.02	0.03	0.01	−0.84 ± 0.03	0.93 ± 0.02	0.01	0.01	0.99	7.1
	M2	−0.26 ± 0.03	0.91 ± 0.03	0.01	0.02	−1.16 ± 0.05	1.20 ± 0.04	0.01	0.01	0.99	16.8
	L2	−0.23 ± 0.03	0.80 ± 0.05	0.38	0.01	−1.00 ± 0.07	1.05 ± 0.07	0.01	0.48	0.95	17.8
GDH	ALL	−1.19 ± 0.03	0.98 ± 0.03	0.01	0.47	−2.14 ± 0.05	1.28 ± 0.04	0.01	0.01	0.93	44.5
	H1	−1.38 ± 0.08	1.07 ± 0.06	0.01	0.28	−2.41 ± 0.13	1.40 ± 0.08	0.01	0.01	0.96	51.5
	M1	−1.29 ± 0.06	1.12 ± 0.08	0.01	0.17	−2.38 ± 0.13	1.47 ± 0.10	0.01	0.01	0.95	30.9
	L1	−1.27 ± 0.07	1.35 ± 0.17	0.01	0.07	−2.57 ± 0.21	1.77 ± 0.22	0.01	0.01	0.85	51.6
	H2	−1.18 ± 0.07	0.90 ± 0.07	0.06	0.22	−2.05 ± 0.14	1.19 ± 0.09	0.01	0.08	0.93	38.0
	M2	−1.13 ± 0.05	0.95 ± 0.05	0.01	0.32	−2.04 ± 0.09	1.24 ± 0.07	0.01	0.01	0.97	30.5
	L2	−1.07 ± 0.03	0.95 ± 0.05	0.01	0.36	−1.99 ± 0.07	1.25 ± 0.07	0.01	0.01	0.97	17.1
NDPK	ALL	1.60 ± 0.05	0.94 ± 0.06	0.01	0.35	−0.52 ± 0.11	1.24 ± 0.08	0.01	0.01	0.76	112.4
	H1	1.16 ± 0.06	0.62 ± 0.04	0.01	0.01	1.76 ± 0.09	0.82 ± 0.06	0.24	0.01	0.95	34.7
	M1	1.24 ± 0.05	0.64 ± 0.07	0.15	0.01	1.87 ± 0.11	0.84 ± 0.09	0.31	0.11	0.91	25.3
	L1	1.35 ± 0.04	0.19 ± 0.10	0.01	0.01	1.59 ± 0.13	0.33 ± 0.14	0.01	0.01	0.38	25.6
	H2	−0.06 ± 0.14	1.37 ± 0.15	0.01	0.03	−1.39 ± 0.27	1.81 ± 0.19	0.01	0.01	0.87	90.0
	M2	1.91 ± 0.16	1.21 ± 0.17	0.02	0.24	−1.08 ± 0.30	1.59 ± 0.22	0.01	0.02	0.84	139.0
	L2	1.93 ± 0.09	1.57 ± 0.18	0.01	0.01	−1.45 ± 0.25	2.06 ± 0.24	0.01	0.01	0.87	74.0
G6Pdh	ALL	−1.40 ± 0.03	0.96 ± 0.03	0.01	0.21	−2.33 ± 0.05	1.25 ± 0.04	0.01	0.01	0.93	46.1
	H1	−1.41 ± 0.06	1.01 ± 0.05	0.01	0.88	−2.38 ± 0.10	1.32 ± 0.06	0.01	0.01	0.97	38.8
	M1	−1.34 ± 0.05	0.98 ± 0.07	0.02	0.66	−2.28 ± 0.11	1.27 ± 0.09	0.01	0.01	0.96	24.8
	L1	−1.29 ± 0.08	1.06 ± 0.21	0.17	0.77	−2.32 ± 0.30	1.39 ± 0.33	0.08	0.27	0.67	71.2
	H2	−1.42 ± 0.04	0.89 ± 0.05	0.01	0.04	−2.29 ± 0.08	1.18 ± 0.06	0.01	0.02	0.97	21.9
	M2	−1.53 ± 0.06	0.99 ± 0.06	0.01	0.84	−2.48 ± 0.10	1.30 ± 0.08	0.01	0.01	0.97	34.5
	L2	−1.45 ± 0.07	0.95 ± 0.14	0.18	0.75	−2.37 ± 0.19	1.25 ± 0.18	0.02	0.20	0.80	53.5

Regression model was log Y = log a + b log X. %SEE represents the standard error (as a percentage) of an estimate using the regression equation. Results of tests of the null hypotheses (H_0) b = 0.75 and b = 1.00 are given as P-values. Culture treatments are concentrations of the alga *Dunaliella salina* (L = 2000 cells · ml^{-1}, M = 10,000 cells · ml^{-1} and H = 50,000 cells · ml^{-1}).

significantly different in either case ($P < 0.48$ for both dry and protein mass data).

Discussion

Artemia growth and development

Because development occurred synchronously regardless of culture treatment, enzyme activities could be compared directly in terms of size without need to consider developmental stages. The substantial independence of development rate from food concentration suggests a "growth at all cost" strategy. This would be essential for organisms such as *Artemia*, inhabiting temporary environments. Upon hatching, a premium would be placed on developing to a reproductive stage, regardless of food environment, in order to produce cysts which could survive desiccation. In the Copepoda, another crustacean group in which growth has been more extensively studied, developmental rates for certain species are substantially determined by temperature, while growth rate is a variable more sensitive to food concentration (Miller and Johnson, 1977; Vidal 1980).

Effects of different body size indices

This study documents differences between dry mass- and protein mass-based scaling exponents. This is clearly due to a change in body composition over the developmental period examined; protein mass increased more rapidly than dry mass. An increase in percentage protein as animals mature has previously been found for *Artemia* (Persoone *et al.*, 1980).

Differences observed between intra- and interspecific exponents may be largely due to differences in body composition. Where the composition of a multispecific group of animals has been successfully standardized (*e.g.*, mammals; see Taylor, 1980), reproducible and comparable scaling exponents have resulted. The relatively low variation in the composition of mammals contrasts with that of invertebrates, such as copepods, where body composition is highly variable (see Mayzaud, 1986). This may explain the many different scaling exponents derived for invertebrates, in general (see Peters, 1983). The traditional 0.75 exponent is applicable only where body size is expressed as wet mass. Clearly other exponents can be found if protein or dry mass are used. Thus, arguments as to the significance of the 0.75 exponent should be reconsidered, particularly in groups of animals where reliable wet mass cannot be determined, or where body composition varies widely or changes with size. Thus, the selection of a mass variable is worthy of at least as much consideration as the standardization of conditions of measurement of a rate variable (*e.g.*, selecting a "basal" metabolic rate for respiration studies). Protein content may offer a suitable index of body size.

Enzyme response to culture treatments

Animals fed identical diets did not respond with identical growth rates. The reasons for this are unclear, but are not of particular concern, because it is the independently measured growth rates resulting from food levels that are of primary interest. For this reason, the term "culture treatment" will be used in discussion to distinguish the growth rate effects from the food level treatment themselves. Thus, the experiment consisted of six culture treatments.

On the basis of regression analyses, enzymes may be placed into three groups: those in which variation in maximal activity across size is completely independent of treatment, those exhibiting some statistical differences between treatments (but neither predictably nor consistently with growth rate), and those showing consistent differences related to culture treatments.

The first group includes ala AT and G6Pdh. No differences in scaling exponents were found between culture treatments. Because growth rates varied between culture treatments (see Tables Ia and b), we can state that the scaling relationships for these enzymes do not appear to be influenced by growth rate.

For the second enzyme group, some statistical differences in scaling exponent between treatments were detected; these include CS, LDH, PK, asp AT, and GDH. These statistical differences usually identified cultures L1 and H2 as distinct. In terms of growth rates, however, neither L1 nor H2 cultures were distinct (based on 95% confidence intervals of the exponent g). Thus, whatever the differences indicate, they do not appear to be related to growth rate.

NDPK alone (the sole member of the third group) showed variation that could be attributed to differences in growth rates. In trial 1 (Fig. 6 A, B), for a given body mass, culture H1 has a higher activity than M1 which is again higher than L1, for both protein and dry mass data. For trial 2, (data not shown) this pattern was less clear because cultures M2 and H2 had similar exponential growth coefficients (Table I), however their enzyme activities were also similar (52 ± 8 units at 100 μg mass). Clearly NDPK activity does not vary *directly* with growth because, within cultures, it changes with body size, whereas the measured specific growth rate is constant (because growth is exponential). Rather, the differences between cultures are related to the changes in enzyme activities with size. These differences do not appear statistically, as differences between regression parameters, due to a clear lack of fit in allometric models.

NDPK activity reached a plateau in all cultures. Therefore, enzyme activity per unit mass must actually *decrease* from the point at which the plateau is reached. The significance of such a plateau in enzyme activity is unclear.

It may be that there is some physiological mechanism for sensing available food resources and altering development to achieve enzyme levels that are sufficient for possible future growth under prevailing conditions. Enzyme activity may simply anticipate a future decline in animal growth rate. Alternatively, it may mark the beginning of a transition from somatic growth to reproduction. It is clear, however, that NDPK scaling is affected by differences in growth rate. NDPK activity may in fact be useful as a predictor of growth rate.

In summary, among eight enzymes representative of both anabolic and catabolic processes, only one (NDPK) showed a relationship between maximal activity and growth rate. We shall elaborate on this relationship elsewhere. The meaning of particular values obtained for individual enzyme scaling exponents will be considered in a separate study (Berges and Ballantyne, unpub.) for a variety of crustacean species.

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Ammonia Induces Settlement Behavior in Oyster Larvae

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Abstract. Oyster larvae exposed to solutions of NH_4Cl exhibit stereotypical settlement behavior similar to that which normally precedes cementation and metamorphosis. Un-ionized ammonia is the active chemical species. At $pH = 8.0$, the threshold concentration of NH_4Cl ($pH = 8.0$) for newly competent larvae is 2.5 m*M;* maximum activity is at 7.9 m*M*, corresponding to calculated NH_3 concentrations of 100 μM and 310 μM, respectively. Induction of settlement behavior is rapid, with >90% of larvae exposed to 310 μM NH_3 responding within less than 5 min. After 15 to 30 min, larvae become habituated to NH_3 and resume swimming so that the percent exhibiting settlement behavior after 30 min is <10%. Other weak bases, such as methylamine and trimethylamine, induce similar behavior suggesting that NH_3 acts by increasing intracellular pH. Evidence that NH_3 and L-3,4-dihyrodxyphenylalanine (L-DOPA) induce settlement behavior through different mechanisms is presented. Ammonia may be a natural environmental cue that promotes oyster settlement behavior and, ultimately, recruitment.

Introduction

Many marine invertebrates, including oysters, have planktonic larvae that are recruited preferentially to habitats suitable for subsequent survival (Thorson, 1950). Recruitment of invertebrate larvae often involves a stereotyped series of search and crawl behaviors that is called settlement, followed by a morphogenetic phase called metamorphosis (Burke, 1983). The settlement behavior of oyster larvae has been well characterized and includes swimming with the foot extended forward followed by a series of increasingly localized crawling maneuvers (Prytherch, 1932; Cranfield, 1973; Coon *et al.*, 1985). If the habitat in which the larva has settled is suitable, the larva will cement permanently to the substratum and metamorphose. Settlement is reversible and does not necessarily culminate in metamorphosis once initiated; if the habitat is unsuitable, the larva may resume swimming and repeat the process elsewhere.

Invertebrate larvae are often induced to settle and metamorphose by environmental cues, typically chemical, associated with the adult habitat (Crisp, 1974; Chia and Rice, 1978). Microbial films play an important role in the development of many invertebrate assemblages (Zobell and Allen, 1935; Meadows and Campbell, 1972; Scheltema, 1974; Bonar *et al.*, 1986). Both soluble and surface-associated bacterial products are important in recruiting invertebrate larvae to surfaces containing bacterial films (Wilson, 1955; Scheltema, 1961; Gray, 1967; Muller, 1973; Neumann, 1979; Kirchman *et al.*, 1982), although some larvae prefer unfilmed surfaces (Crisp and Ryland, 1960). A bacterium, *Alteromonas colwelliana* (originally called LST), was found to enhance the recruitment of oyster larvae to colonized substrates (Weiner *et al.*, 1985; 1989). Supernatants from cultures of *A. colwelliana*, as well as other bacteria, contain one or more soluble factors that induce settlement behavior in oyster larvae of the genus *Crassostrea*. Preliminary studies showed that the soluble inducer has a low molecular weight (<300 daltons), and that supernatants have increased inductive potency commensurate with the age of the bacterial culture (Fitt *et al.*, 1990).

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Experiments reported in this paper demonstrate that solutions of NH_4Cl induce settlement behavior, and that NH_3, not NH_4^+, is the active chemical species. Additional experiments further explore the relationship between the mechanism of NH_3 induction and induction of settlement behavior by L-3,4-dihydroxyphenylalanine (L-DOPA), another known soluble inducer of oyster settlement behavior (Coon *et al.*, 1985, 1990). Preliminary results of this work have been presented (Coon *et al.*, 1988; Bonar *et al.*, 1990).

Materials and Methods

Obtaining and maintaining larvae

Larvae of the Pacific oyster, *Crassostrea gigas*, were obtained from the Coast Oyster Company of Quilcene, Washington, and maintained in the laboratory (Coon *et al.*, 1990). Larvae were used within one week of arrival.

Bioassay procedure

Experiments were conducted as previously described (Coon *et al.*, 1990). Aliquots of 20–50 larvae were assayed in 24-well tissue culture plates (Falcon #3047) in a final volume of 1.0 ml. Antibiotics were not used, but all experiments were conducted in 0.2 μm filtered seawater. Each treatment was duplicated or triplicated, and results are expressed as the mean ± standard error. All chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri).

Larval settlement behavior was defined as in Coon *et al.* (1990), the basic criterion being active foot extension beyond the ventral margin of the shell. Behavior in each well was monitored with a dissecting microscope for 30 s at the times noted. The length of each experiment was between 30 and 40 min, as noted.

Statistical tests were performed on arcsine-transformed data using a one-way analysis of variance (ANOVA) within time points. The ANOVA was followed by a Student-Newman-Keuls pair-wise comparisons test when significant differences were detected (Zar, 1974). Differences were considered significant if $P < 0.05$.

Effects of ammonia, ammonium, and pH

Larvae were exposed to a range of concentrations of NH_4Cl, in the first series of experiments. Stock solutions of NH_4Cl were made in seawater at twice the final concentration and adjusted to pH = 8.0 with NaOH. At the beginning of each bioassay, 0.5 ml of stock solution was added to an equal volume of seawater (pH = 8.0) containing swimming larvae. This experiment was repeated using $(NH_4)_2SO_4$ and other chloride salts (NaCl, KCl) at concentrations up to 10 m*M*.

Approximately 96% of the total ($NH_3 + NH_4^+$) in seawater at pH = 8.0 is present as the ammonium ion, NH_4^+ (Bower and Bidwell, 1978). To determine whether NH_3 or NH_4^+ was the active chemical species, larval settlement responses were observed while the concentrations of NH_3 and NH_4^+ were varied under two different regimes. In the first, pH was held constant at 8.0 and the total ($NH_3 + NH_4^+$), as NH_4Cl, was varied as described for the initial experiments above. In the second regime, total ($NH_3 + NH_4^+$) was held constant at 5.0 m*M* and the proportion of NH_3 to NH_4^+ was varied by altering the pH. The absolute concentrations of NH_3 and NH_4^+ were calculated by means of a hydrolysis constant for ammonium ion in seawater of $pK_a^s = 9.39$, at 30‰ salinity, 23°C and 1 atm pressure (Bower and Bidwell, 1978).

Because ammonia is a weak base ($pK_a = 9.25$), its effects might result from an increase in intracellular pH (pH_i). Therefore, two other weak bases, methylamine ($pK_a = 10.7$) and trimethylamine ($pK_a = 9.81$), were tested for their ability to induce settlement behavior. The inductive activities of these two compounds, along with those of NH_4Cl, were investigated according to the original protocol described above; concentration was varied while the pH remained constant at 8.0.

Relationship of NH_3-induction to L-DOPA-induction of settlement behavior

To determine whether NH_3 and L-DOPA induce settlement behavior through the same mechanisms, we tested sulpiride, a dopaminergic receptor antagonist (Stoof and Kebabian, 1984) and potent inhibitor of L-DOPA-induced settlement behavior (Coon and Bonar, 1987), for its ability to block NH_3-induced settlement behavior. Ammonium chloride stock solutions were made 10 times the final concentration in filtered seawater and adjusted to pH = 8.0. Solutions of L-DOPA and sulpiride were made 10 times their final concentrations in 0.002 *N* HCl. All larvae were pre-incubated in either 100 μM sulpiride or seawater for 12 min, then exposed to either 10 m*M* NH_4Cl or 100 μM L-DOPA. The effects of seawater and 0.002 *N* HCl were appropriately controlled. The pH of the final solutions was 7.7, yielding a calculated NH_3 concentration of 270 μM.

In other experiments, larvae that were "habituated" to NH_3 (see Results) were tested to see whether they would still respond to L-DOPA. Larvae were exposed to 5.0 m*M* NH_4Cl for 18 min until they began to habituate. They were then removed, rinsed, and exposed to either: (1) 100 μM L-DOPA; (2) fresh 5.0 m*M* NH_4Cl; (3) the NH_4Cl solution from which they had just been removed; or (4) filtered seawater. Control groups were pre-exposed to filtered seawater instead of NH_4Cl, then rinsed and put in

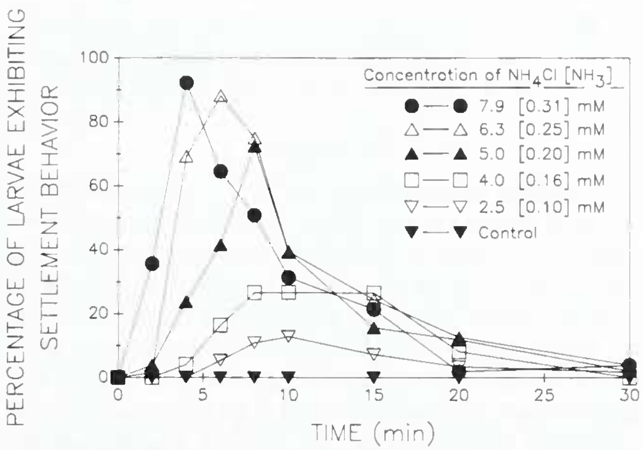


Figure 1. Percentages of *Crassostrea gigas* larvae exhibiting settlement behavior as a function of length of time exposed to specified concentrations of NH_4Cl at pH = 8.0. Calculated NH_3 concentrations are shown in brackets for reference. Data are means of duplicates followed through time. Controls contained only filtered seawater (pH = 8.0).

the same four treatments. In addition, some larvae were left in the NH_4Cl solution without rinsing, and were exposed to either: (1) the addition of another 5.0 m*M* NH_4Cl; (2) the addition of 100 μM L-DOPA; or (3) no additional treatment. Larval settlement behavior was monitored for an additional 39 min.

Results

Ammonium chloride induces settlement behavior

Ammonium chloride induced high levels of settlement behavior in oyster larvae (Fig. 1). The percentage of larvae exhibiting settlement behavior reached >90% within 5 min of exposure to an NH_4Cl solution of 7.9 m*M* at pH = 8.0. Responses to higher concentrations of NH_4Cl are not shown because larvae in these solutions exhibited reduced activity levels after short exposures. Between 2.5 and 7.9 m*M* (pH = 8.0), the larval response to NH_4Cl was concentration dependent. As the NH_4Cl concentration increased, the percentage of larvae exhibiting settlement behavior increased, and the length of time required for the maximum percentage of larvae to respond decreased. Following the maximum larval response, the percentage of larvae continuing to exhibit settlement behavior rapidly declined so that 30 min after the initial exposure, almost all the larvae had "habituated" to the NH_4Cl solutions and had resumed normal swimming. No subsequent metamorphosis was observed after 24 to 48 h.

Larvae also exhibited high levels of settlement behavior in response to $(NH_4)_2SO_4$, indicating that either NH_3 or NH_4^+ was the active chemical species. This was corroborated by the observation that larval settlement behavior was not induced by Cl^-—as NaCl or KCl—at concentrations comparable to inductive NH_4Cl solutions (data not shown). Methylamine and trimethylamine, which are weak bases like NH_3, induced high levels of oyster settlement behavior (Table I).

The active species is NH_3 rather than NH_4^+

Ammonia has a pK_a[5] of 9.39 in seawater and its calculated speciation as a function of pH is shown in Figure 2A. As the pH of the NH_4Cl solution decreases from 8.0 to 7.0, which is within the physiological tolerance range for oyster larvae, the NH_3 concentration changes much more dramatically (89.6% decrease) than the NH_4^+ concentration (3.6% increase) (Fig. 2B). Theoretically, the chemical species, NH_3 or NH_4^+, to which the larvae are responding, would have the same dose-response curve, whether the concentrations are adjusted by varying the NH_4Cl concentration under constant pH, or by keeping the NH_4Cl concentration constant and varying the pH.

This experiment shows that the maximal percentage of larvae exhibiting settlement behavior in response to NH_3 was independent of the regime used to vary the NH_3 concentration (Fig. 3A). The difference between these two curves represents less than 0.1 pH unit, which was within experimental error. In contrast, the larval response to NH_4^+ was highly dependent on the regime used to vary the NH_4^+ concentration; the larval response increased with increasing NH_4^+ concentration when the pH was held constant while the NH_4Cl concentration was varied, but the larval response decreased with increasing NH_4^+ concentration when the NH_4Cl was held constant while the pH was varied (Fig. 3B). These results indicate that, in these solutions, NH_3, not NH_4^+, was the active chemical species inducing settlement behavior in oyster larvae.

NH_3 and L-DOPA induce settlement behavior through different mechanisms

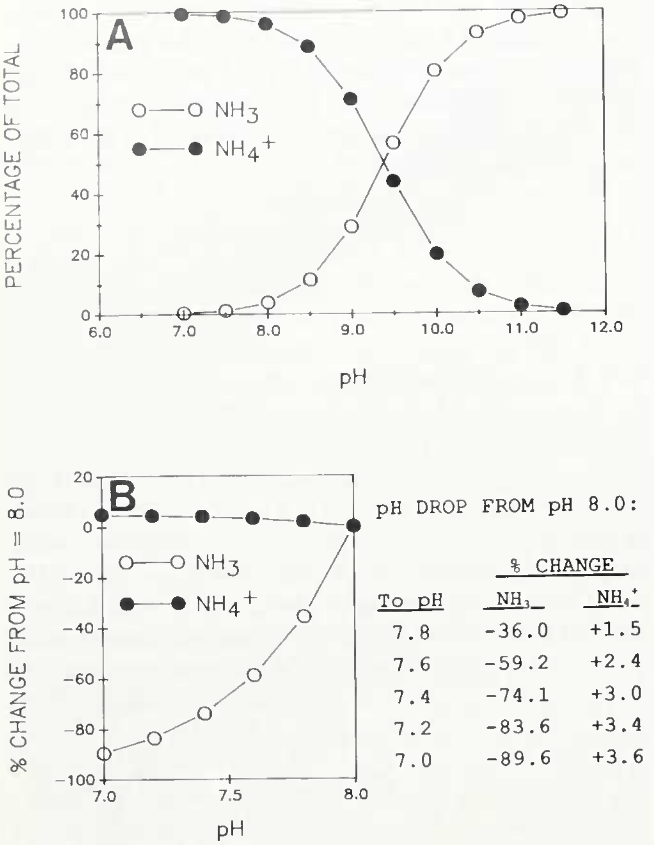
The dopaminergic antagonist, sulpiride, blocked the ability of L-DOPA to induce settlement behavior (Fig.

Table I

Maximal percentage of oyster larvae exhibiting settlement behavior in response to exposure to weak bases at pH = 8.0

	pK_a	1.0 m*M*	3.3 m*M*	10 m*M*
NH_4Cl	9.25	3.2 ± 0.4	51.2 ± 4.0	90.3 ± 2.3
Methylamine	10.7	4.4 ± 1.4	47.6 ± 14.2	94.6 ± 2.4
Trimethylamine	9.81	0	19.7 ± 7.6	91.6 ± 5.4

Data are means of duplicates ± standard error.



To pH	NH_3	NH_4^+
7.8	-36.0	+1.5
7.6	-59.2	+2.4
7.4	-74.1	+3.0
7.2	-83.6	+3.4
7.0	-89.6	+3.6

Figure 2. Calculated effect of pH on NH_3 and NH_4^+ speciation. (A) Percentage contribution to the total (NH_3 + NH_4^+) by each species as a function of pH. $pK_a^s = 9.39$. (B) Percentage change in NH_3 and NH_4^+ as the pH of the solution drops from pH = 8.0 to the specified value. Actual calculated changes in speciation are tabulated for clarity.

4A) but did not block the ability of NH_3 to induce settlement behavior (Fig. 4B). However, two small effects of sulpiride on NH_3-induced settlement behavior were noted: (1) settlement behavior was more rapidly expressed; and (2) the maximum percentage of larvae induced to exhibit settlement behavior was slightly lower (*t*-test; $P < 0.1$). The differential effects of sulpiride on the abilities of NH_3 and L-DOPA to induce settlement behavior indicate that NH_3 functions through a mechanism that does not require the dopaminergic receptor involved in the induction of settlement behavior by L-DOPA (Coon and Bonar, 1987).

The effects of NH_3 and L-DOPA are not completely independent. Although larvae that had habituated to NH_3 were almost completely refractory to fresh NH_3, they could still respond to L-DOPA (Fig. 5). However, fewer of these larvae exhibited settlement behavior, and they responded more slowly to L-DOPA than larvae that had not been habituated to NH_3. The larvae also showed an attenuated response to L-DOPA in additional treatments during which they were left in the presence of NH_3 when L-DOPA was added (data not shown). The small, transient, increase in settlement behavior following transfer to a new NH_4Cl solution was an artifact of the procedure.

Discussion

This study demonstrates that NH_3 in the surrounding medium induces oyster larvae to exhibit settlement behavior. The onset of settlement behavior is rapid, high percentages of larvae are induced to behave, and the larvae quickly resume swimming without cementing to the plastic culture plates (a suboptimal settlement surface) in which the experiments were conducted. The results also indicate that, although NH_3 and L-DOPA induce similar settlement behaviors, the biochemical mechanisms by which they do so are different.

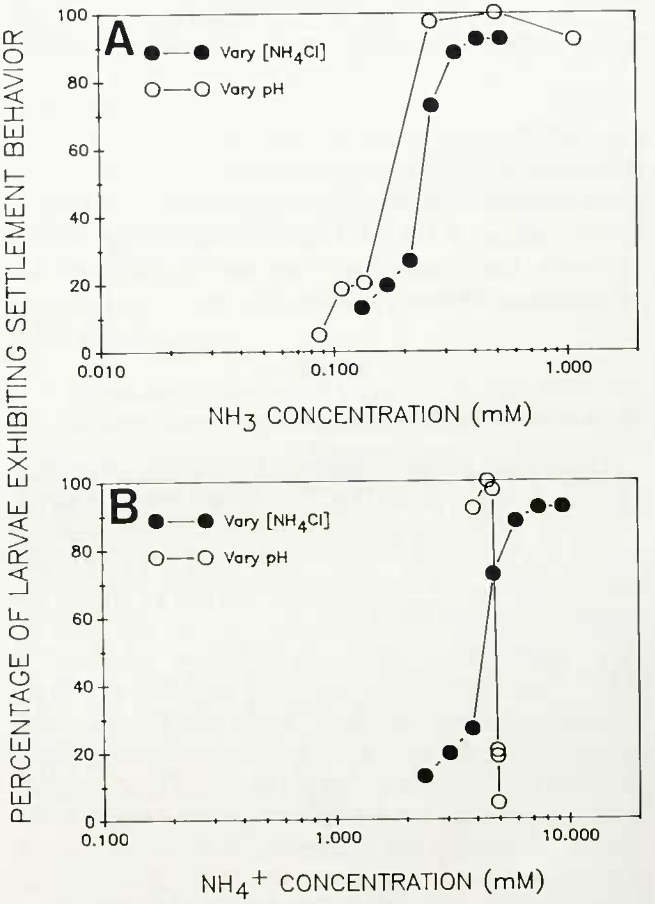


Figure 3. Maximal percentages of *Crassostrea gigas* larvae exhibiting settlement behavior as a function of the concentration of either NH_3 or NH_4^+. In one regime, pH was held constant while the NH_4Cl concentration was varied (data calculated from Fig. 1); in the other regime, NH_4Cl concentration was held constant while the pH was varied. The concentrations of NH_3 and NH_4^+ were calculated from pH values and NH_4Cl concentration. (A) Larval response as a function of the calculated concentrated of NH_3 under the two regimes. (B) Larval response as a function of NH_4^+ under the two regimes. Data are means of duplicates.

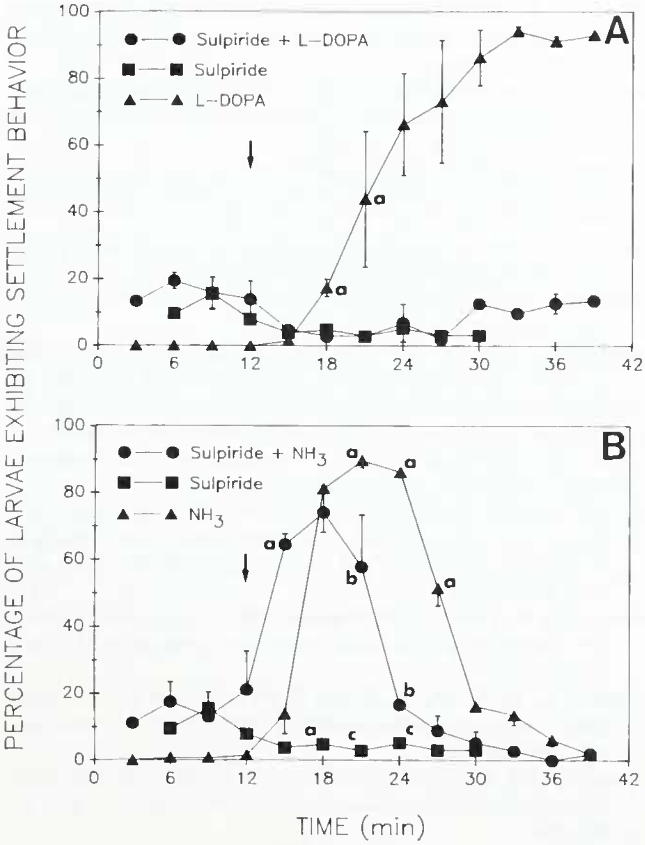


Figure 4. Percentages of *Crassostrea gigas* larvae exhibiting settlement behavior as a function of length of the duration of their exposure to NH_3 or L-DOPA in the presence of sulpiride. Sulpiride is a dopaminergic receptor antagonist. (A) Effect of sulpiride on the ability of L-DOPA to induce settlement behavior. (B) Effect of sulpiride on the ability of NH_3 to induce settlement behavior. Larvae were pre-incubated in sulpiride (100 μM) or seawater for 12 min, then L-DOPA (100 μM), NH_4Cl (10 mM), sulpiride (100 μM) or seawater were added as indicated by the arrows. Data are means ± standard error of triplicates followed through time. For each time point, treatments with the same letter, or no letter are not significantly different from each other. Only statistics for relevant time points are shown for clarity.

Ammonia, as a by-product of protein catabolism, is excreted by most marine bacteria and animals (Campbell, 1970; Billen, 1984). Therefore, in areas of high biological activity and reduced mixing (such as in boundary layers near surfaces), NH_3 might reach levels high enough to induce settlement behavior in oyster larvae. Total (NH_3 + NH_4^+) concentrations of 10 mM have been reported in interstitial waters from marine sediments (Bruland, 1983). Stevens (1983) found that total (NH_3 + NH_4^+) concentrations in association with oyster reefs may reach greater than 200 μM in sediment waters and 3 μM in overlying waters 10 cm above the sediment interface. The presence of high levels of NH_3 in the environment, the rapid induction of settlement behavior by NH_3, and the quick reversibility of inductive effects of NH_3, strongly suggest that NH_3 is a natural environmental cue for recruitment of oyster larvae. Its actual involvement in larval recruitment, however, has not yet been demonstrated.

If NH_3 is a natural inducer of settlement behavior, then it must be a relatively non-specific indicator of biologically rich environments. Ammonia alone could not account for the specificity observed in natural oyster settlement and metamorphosis. We hypothesize that NH_3 acts as a chemokinetic agent that induces settlement behavior once a threshold concentration is encountered, bringing oyster larvae into contact with substrates and other potential contact-dependent and soluble cues (*c.f.* Crisp, 1974). Once settlement behavior has been initiated, oyster larvae rely on other cues from the environment to indicate that the habitat is suitable for cementation and metamorphosis. If these secondary cues are not present, the larvae habituate to NH_3 and swim away. This scenario is consistent with models and observations of oyster settlement (Prytherch, 1934; Cranfield, 1973; Coon *et al.*, 1985; Weiner *et al.*, 1989; Coon *et al.*, 1990).

Although the mechanism by which NH_3 induces settlement behavior in oysters is unknown, NH_3 acts by increasing pH_i in other invertebrate systems (Boron and DeWeer, 1976; Roos and Boron, 1981; Dube and Guerrier, 1982; Ward *et al.*, 1983; Bibring *et al.*, 1984; Williams *et al.*, 1984; Busa, 1986). Weak bases, such as NH_3, raise pH_i by penetrating the cell membrane as the uncharged

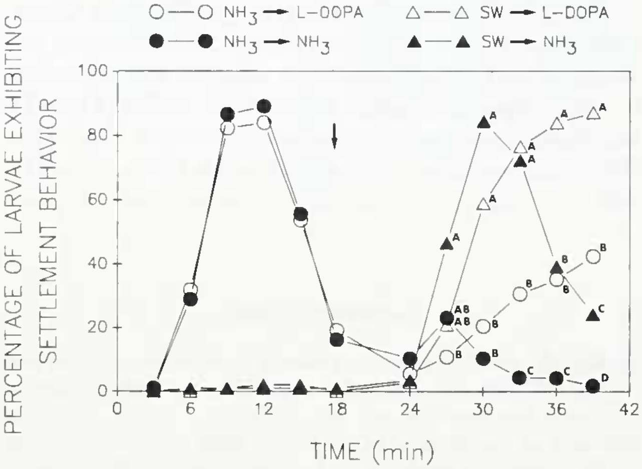


Figure 5. Percentages of *Crassostrea gigas* larvae exhibiting settlement behavior as a function of length of time exposed to various regimes of NH_4Cl and L-DOPA. Larvae were exposed to either NH_4Cl (5 mM) or seawater for 18 min, then removed (downward pointing arrow) and put into either NH_4Cl (5 mM) or L-DOPA (100 μM). Data are means ± standard error of triplicates followed through time. For each time point, treatments with the same letter, or no letter, are not significantly different from each other. Only statistics for relevant time points are shown for clarity.

species, then reprotonating in the cytoplasm (Roos and Boron, 1981). The induction of settlement behavior by other weak bases, such as methylamine and trimethylamine, is consistent with NH_3 acting by increased intracellular alkalization. An increase in pH_i would not be expected to be cell-type specific and so may affect a diverse range of cell types in the larvae. Larvae of the hydroid, *Hydractinia*, are induced to metamorphose by NH_4^+, not NH_3, through a mechanism that may involve regulation of intracellular transmethylation rather than pH_i (Berking, 1988).

Whatever its mode of action, induction of settlement behavior by NH_3 clearly involves a mechanism different from that of L-DOPA induction, though they are probably related. Ammonia and L-DOPA have different time courses. Larvae respond quickly to NH_3, then soon habituate to it; in contrast, larvae respond more slowly to L-DOPA, and the effects are longer lasting. Induction of settlement behavior by NH_3 is not mediated through the same dopaminergic receptors required for induction by L-DOPA, but is effected slightly by blocking these receptors with sulpiride. Conversely, larvae habituated to NH_3 can still respond to L-DOPA but to a lesser degree. There may be some interaction between pH_i and signal transduction through the dopaminergic receptors. Further experiments are underway to resolve the mechanism of NH_3-induction.

Acknowledgments

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Induction of Metamorphosis of Larvae of the Green Sea Urchin, *Strongylocentrotus droebachiensis*, by Coralline Red Algae

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Abstract. The coralline red algae, *Lithothamnion glaciale, Phymatolithon laevigatum, P. rugulosum*, and *Corallina officinalis*, induced >85% of laboratory-reared larvae of *Strongylocentrotus droebachiensis* to metamorphose. Larvae must contact live *L. glaciale* or its spores for metamorphosis to occur; the inducer is not sensed in the water column. However, aqueous extracts of *L. glaciale* can induce metamorphosis, suggesting that the inducing factor is chemical. Neither ashed nor boiled *L. glaciale* induces metamorphosis, indicating that the factor is heat-labile and that thigmotaxis, per se, is not important in the response. The amino-acid, γ-aminobutyric acid (GABA), which induces settlement of other marine invertebrate larvae, also induces significant rates of metamorphosis of *S. droebachiensis* at concentrations $\geq 10^{-4}$ *M*. A reduction (with antibiotics) in the number of live bacteria on the surface of *L. glaciale* does not affect the rate of metamorphosis of larvae.

Introduction

The larvae of a variety of benthic marine invertebrates are known to settle and metamorphose in response to coralline red algae, including: corals, *Agaricia agaricites danai, A. agaricites humilis*, and *A. tenuifolia* (Morse *et al.*, 1988); chitons, *Tonicella lineata* (Barnes and Gonor, 1973), *Mopalia muscosa* (Morse *et al.*, 1979a), and *Katharina tunicata* (Rumrill and Cameron, 1983); limpets, *Acmaea testudinalis* (Steneck, 1982); trochid gastropods, *Trochus niloticus* (Heslinga, 1981); abalone, *Haliotis* spp. (Shepherd, 1973; Morse *et al.*, 1979a, 1980a; Morse and Morse, 1984; Shepherd and Turner, 1985); tubeworms, *Spirorbis corallinae* (de Silva, 1962) and *S. rupestris* (Gee, 1965); sea urchins, *Strongylocentrotus purpuratus* (Rowley, 1989); and seastars, *Acanthaster planci* (Henderson and Lucas, 1971; Yamaguchi, 1973; Lucas and Jones, 1976) and *Stichaster australis* (Barker, 1977).

The relationship between some grazers and coralline algae may be mutually beneficial, and the species may have co-evolved. For example, by preferentially settling and metamorphosing on crustose coralline algae, the abalone *Haliotis rufescens* gains obligate chemical cues for the induction of metamorphosis and further development, micro-refuges from predation, adequate food to support early growth (*e.g.*, mucous exudates of the coralline alga, diatoms, bacteria, and other epiphytes), and camouflage (the red pigment of the coralline alga is incorporated into the shell of the developing abalone). In turn, the coralline alga is cleaned of epiphytic algae (which reduce photosynthesis and can potentially kill the coralline) by the abalone's grazing activity (Morse *et al.*, 1980a; Morse and Morse, 1984). A similar, mutualistic relationship has been shown with the limpet, *Acmaea testudinalis*, and the coralline alga, *Clathromorphum circumscriptum* (Steneck, 1982).

In the shallow rocky subtidal zone of north temperate oceans, strongylocentrotid sea urchins are generally associated with coralline algal-dominated communities, described by various workers as "barren grounds" (Pearse *et al.*, 1970; Lawrence, 1975), "Isoyake areas" (Hagen, 1983), or "coralline flats" (Ayling, 1981). In many cases, the destructive grazing of kelps and other fleshy macroalgae by expanding populations of sea urchins has led to the establishment of these coralline communities, which are maintained by continued intensive grazing (see reviews by Lawrence, 1975; Lawrence and Sammarco, 1982; and Chapman, 1986). This has been well documented for *Strongylocentrotus droebachiensis* in eastern Canada

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(Mann and Breen, 1972; Breen and Mann, 1976a, b; Lang and Mann, 1976; Mann, 1977; Breen, 1980; Chapman, 1981; Wharton and Mann, 1981), where the common shallow-water species of coralline algae are *Clathromorphum circumscriptum, Corallina officinalis, Lithothamnion glaciale, Phymatolithon laevigatum,* and *P. rugulosum.* Recruitment of *S. droebachiensis* (Lang and Mann, 1976; Wharton and Mann, 1981; Miller, 1985; Scheibling, 1986) and other strongylocentrotid species (Pearse *et al.,* 1970; Tegner and Dayton, 1981) is lower in kelp beds than in coralline barren grounds, and selective settlement of sea urchin larvae on coralline substrata may account, at least in part, for these differences (Raymond and Scheibling, 1987).

In this study, we show that larvae of *S. droebachiensis* are induced to settle and metamorphose in the presence of coralline algae. In a series of laboratory experiments with *L. glaciale,* we investigate the potential mechanism of settlement induction. We discuss the implications of this result to settlement patterns in the field.

Materials and Methods

Larval rearing

Adults of *Strongylocentrotus droebachiensis* were collected at 5–10 m depth at Sandy Cove (Digby County), Nova Scotia, Canada (44° 29′ N, 66° 05′ W). They were maintained in the laboratory in running seawater and fed kelp (*Laminaria digitata* and *L. longicruris*) at regular intervals.

Gametes from adults of *S. droebachiensis* (50–85 mm test diameter) were obtained by peristomial injection of 2.5–4.0 ml of 0.53 *M* KCl. Females shed their eggs into glass bowls of chilled 0.45 μm Millipore®-filtered seawater (hereafter referred to as filtered seawater); males shed sperm into dry, chilled bowls. After ~20 min of spawning, the eggs were rinsed three to four times with filtered seawater. Several drops of sperm (checked under a microscope for motility) from one male were mixed with the eggs from one female for ~10 min. The eggs were then rinsed another three to four times with filtered seawater. Mean (±SD) percentage of fertilized eggs, as judged by the presence of a fertilization membrane, was 99.4 ± 0.6% (n = 15).

Early-stage embryos were reared in standing cultures in small glass bowls for ~72–120 h post-fertilization. When blastulae were seen swimming at the surface of the water, they were transferred to 4-l glass jars containing ~3 l of filtered seawater which was stirred constantly by T-paddles attached to 10-rpm motors. Larval densities, after the first week in stirred cultures, were maintained at ≤2 individuals ml^{-1}. All culturing was carried out in filtered seawater at 10.8 ± 1.4°C (mean ± SD, n = 535), approximating ambient seawater temperatures off Nova Scotia in June and July when larvae of *S. droebachiensis* are settling (Raymond and Scheibling, 1987). Fluorescent lighting provided a light intensity (at culture jar level) of 99.6 ± 11.6 $\mu E\ m^{-2} s^{-1}$ (mean ± SD, n = 3) on a 12 L:12 D photoperiod. Every second day (occasionally every third day) 50–75% of the culture water was removed by reverse filtration and replaced with fresh filtered seawater and microalgal food. The larvae were fed *Dunaliella tertiolecta* (a unicellular green alga) at a concentration of 1×10^4 cells ml^{-1} of culture water. Algae were cultured at 23°C under constant fluorescent illumination in f/2 nutrient medium (Guillard and Ryther, 1962). Only larvae that were deemed competent were used in experiments. Competence was indicated by the presence of large juvenile rudiments and a high rate of metamorphosis (>60%) in trial assays with coralline algae. The time from fertilization to competency ranged from 33 to 51 days.

Experimental protocols

For any experiment, only larvae from the same batch were used. If more than one culture jar of larvae was required for an experiment, larvae from different jars were thoroughly mixed before allocation to treatments. Experiments were run in 250-ml glass jars with ~150 ml of filtered seawater and a test substratum or ~150 ml of a test solution. Five replicate jars (each with 25 larvae) were used per treatment (except where noted). Larvae were transferred into experimental jars with a syringe. Experiments were run for ~24 h (range: 24–28 h) in the same environmental chamber and at the same temperature and photoperiod as larval cultures. The light intensity at experimental jar level (shaded during the light period) was 2.8 ± 0.1 $\mu E\ m^{-2} s^{-1}$ (mean ± SD, n = 3).

After 24 h, larvae and recently metamorphosed juveniles were located in jars using a dissecting microscope and classified as: (1) free-swimming (larvae only), (2) on test alga (when an algal substratum was present), or (3) on bottom or sides of experimental jar. To facilitate the location of recently metamorphosed individuals (221–392 μm test diameter) on coralline algal substrata, the following technique was used. After counting and removing any free-swimming larvae from a jar, the coralline algal substratum was removed and immersed in an isotonic solution of MgCl in water (72 g ml^{-1}) to narcotize any juveniles or larvae on the alga. These would then easily be displaced by gentle agitation or washing of the substratum. In some cases, 3–5 ml of buffered 10% formalin in seawater were added to the MgCl samples so that counting could be postponed. Because counting was time consuming (requiring 2–14 h), replicates were set up in a completely randomized block design, and blocks of treatments were counted in succession. However, only one experiment (with coralline algal extract) had a significant block effect, indicating that the majority of larvae that metamorphosed did so during the experimental period and not during subsequent counting of individuals.

Two controls were used for each experiment: (1) filtered seawater without any test substratum, to ensure that larvae were not metamorphosing in response to handling procedures or other unknown factors, and (2) a cobble encrusted with *Lithothamnion glaciale* (occasionally *Phymatolithon laevigatum* or *P. rugulosum*), to assess the proportion of larvae capable of metamorphosing, because the rate of metamorphosis is generally maximal in response to coralline algae (see Results).

The rate of metamorphosis was expressed as the number of individuals metamorphosed divided by the total number of individuals recovered (n) (usually ≥90% of individuals were recovered). An individual was scored as metamorphosed if the larval arms had been resorbed and the globular test, tube feet, and spines of the juvenile were apparent.

To compare the rate of metamorphosis in response to morphologically different types of coralline red algae, *Corallina officinalis* (finely branched, arborescent form), *Lithothamnion glaciale* (rugose, crustose form), and *Phymatolithon laevigatum* or *P. rugulosum* (smooth, crustose forms) (the latter two species were not distinguished and hereafter are referred to collectively as *Phymatolithon*) were collected subtidally at Eagle Head (44° 04′ N, 64° 36′ W) and Mill Cove (44° 36′ N, 64° 04′ W), Nova Scotia. *Lithothamnion glaciale* and *Phymatolithon* were collected as monocultures totally encrusting cobbles. Cobble sizes were: length, 34.6–56.4 mm; width, 24.0–44.1 mm; height, 12.9–38.5 mm. Tufts of *C. officinalis*, of similar dimensions, were presented upright in experiments. Algae were immediately transported to the laboratory in coolers where they were maintained in separate 91 × 61 × 45 cm fiberglass aquaria with running seawater. All algae were carefully cleaned of epibionts and debris and thoroughly rinsed with filtered seawater prior to use in experiments. *Lithothamnion glaciale* and *Phymatolithon* also were scrubbed with a stiff plastic brush.

To examine the effect of surface contour, in the absence of living tissue, on metamorphosis of *S. droebachiensis*, *L. glaciale* was killed either by ashing at 500°C for 4 h in a muffle furnace or by vigorous boiling in deionized water for two 15-min periods. Killed *L. glaciale* was washed in running seawater prior to experimental use (ashed for 7 days, boiled for 30 min).

To test whether *L. glaciale* released a chemical into the water that could induce metamorphosis of free-swimming larvae, five cobbles encrusted with *L. glaciale* were placed in 2 l of filtered seawater in the environmental chamber for 24 h. The *Lithothamnion*-conditioned filtered seawater was then decanted and used in an experiment with filtered seawater and *L. glaciale* controls.

To test whether urchin larvae metamorphosed in response to a diffusion gradient of inducer molecules surrounding *L. glaciale*, treatments with *Lithothamnion*-encrusted cobbles conducted under static and agitated (on a shaker table at 126 rpm) conditions were compared (this was the lowest possible speed of rotation capable of totally dispersing 1 ml of concentrated methylene blue dye in 150 ml of fresh water in under 10 min in test trials).

To test whether a water-soluble extract of *L. glaciale* would induce metamorphosis of urchin larvae, fragments of the alga were chiselled off of cobbles, scrubbed with a brush, and washed with seawater. Four hundred grams of cleaned *L. glaciale* were finely ground up in 800 ml of filtered seawater (at 11–15°C) with a mortar and pestle. The supernatant was decanted and refrigerated overnight at ~4°C, then centrifuged at 27138 × *g* for 10 min at 2–3°C to remove particulates. To test whether larvae responded in a concentration-dependent manner, this supernatant was then serially diluted to 1:5, 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000 with filtered seawater. These dilutions were left overnight in the environmental chamber, and the following day 150 ml of each dilution were added to experimental jar replicates along with larvae. Protein concentration of the undiluted crude extract, as measured at the onset of the experiment using a Sigma Diagnostics® micro-protein determination kit, was 305 μg ml^{-1}.

The amino-acid neurotransmitter, γ-aminobutyric acid (GABA) is known to induce settlement of several benthic marine invertebrates including the chitons, *Mopalia muscosa* (Morse *et al.*, 1979a) and *Katharina tunicata* (Rumrill and Cameron, 1983), and several species of abalone of the genus *Haliotis* (*c.f.* Morse, 1984); GABA-mimetic molecules, present in coralline red algae, have been shown to be the inducers of metamorphosis in *H. rufescens* (Morse *et al.*, 1979a, b, 1980b; Morse and Morse, 1984; Morse, 1985). To test whether induction of metamorphosis of urchin larvae by coralline algae could also be mimicked by GABA, solutions of GABA (obtained from the Sigma Chemical Company) were prepared in filtered seawater and tested for their ability to induce metamorphosis of larvae of *S. droebachiensis* over the concentration range of 10^{-7}–10^{-1} *M*; 10 larvae per replicate were tested.

During an experiment, *Lithothamnion glaciale* occasionally released minute spores (mean diameter ± SD: 109 ± 16 μm, n = 250) that were found on the bottom of jars. To test whether these spores could induce metamorphosis of larvae, a *Lithothamnion*-encrusted cobble was placed in each of 20 experimental jars with filtered seawater for 78 h and the spores collected. The five jars with the most spores (>50) were rinsed three to four times with filtered seawater (spores stayed attached to glass) and

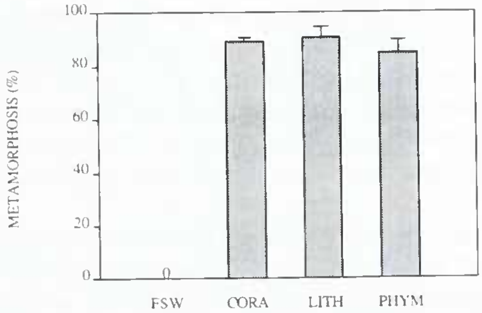

Figure 1. Mean percentage of *Strongylocentrotus droebachiensis* larvae that metamorphosed in response to filtered seawater (FSW) and the coralline red algae, *Corallina officinalis* (CORA), *Lithothamnion glaciale* (LITH), and *Phymatolithon* (PHYM). Each treatment consists of 5 replicates with 25 larvae per replicate. Error bars indicate standard error. 0 denotes that no metamorphosed individuals were found.

used as a treatment in an experiment with filtered seawater and *L. glaciale* controls.

To test whether a reduction in the number of live bacteria on the surface of *L. glaciale* would reduce the rate of metamorphosis of larvae, five *Lithothamnion*-encrusted cobbles were scrubbed with a brush, rinsed with filtered seawater, and put in 1 l of unfiltered seawater containing a mixture of penicillin and streptomycin (1000 units ml^{-1} each). After 42 h, the cobbles were removed (with sterile gloves) and rinsed with filtered seawater to remove the antibiotics and dead bacteria. Bacterial samples were collected from three antibiotic-treated cobbles and three untreated cobbles (which had been similarly scrubbed and rinsed) by swabbing a 1-cm^2 area twice (for 1 min each) with a cotton swab. The adherent material was suspended in 5 ml of artificial seawater and serially diluted in artificial seawater before being plated on marine agar plates. Bacterial colonies were counted after 5 days of development at room temperature. After swabbing, the cobbles were placed in filtered seawater (antibiotic-treated and untreated pieces in separate containers) and left overnight in the environmental chamber before use in the experiment.

All statistical tests were carried out on arcsine-transformed data. This transformation helped to normalize the data and reduce heteroscedasticity. Replicates that had 0/n (no) or n/n (all) larvae metamorphosed were replaced with values of 1/4n and 1–1/4n, respectively, to improve the transformation (Bartlett, 1937). Normality was judged by examination of cumulative probability plots, and heterogeneity of variances was assessed with Cochran's test ($\alpha = 0.01$). All statistical analyses were carried out with the SYSTAT™ (Wilkinson, 1986) statistical computer package. Untransformed values are presented in graphs.

Results

Larvae of *Strongylocentrotus droebachiensis* showed similar, high rates of metamorphosis in response to three morphologically different coralline algae: *Corallina officinalis, Lithothamnion glaciale*, and *Phymatolithon* (Fig. 1). Differences in mean rates among coralline treatments (range: 85–91%) were not statistically significant ($F_{2,12} = 0.45$, $P > 0.05$), indicating that morphology does not affect metamorphic rate under static laboratory conditions. No larvae metamorphosed in a concurrent filtered seawater control, indicating the requirement for an external cue.

The mean rate of metamorphosis of *S. droebachiensis* in response to live *L. glaciale* did not differ significantly among different batches of larvae from different parentage (range: 62–98%, grand mean ± SE: 86.9 ± 2.6%, n = 18) (Kruskal-Wallis test, $P > 0.05$). There also was no significant difference among these batches of larvae in their response to concurrent filtered seawater controls (range: 0–10%, grand mean ± SE: 2.3 ± 0.6%, n = 18) (Kruskal-Wallis test, $P > 0.05$).

In experiments with *L. glaciale*, killing the coralline alga markedly reduced the numbers of metamorphosing larvae (Fig. 2). The rate of metamorphosis with ashed *L. glaciale* was less than a tenth of that with live *L. glaciale*, although it was significantly greater than that in a filtered seawater control (Mann-Whitney U-test, $P < 0.05$). There was no significant difference in the rate of metamorphosis between the filtered seawater control and boiled *L. glaciale* (Mann-Whitney U-test, $P > 0.05$). Neither ashing nor boiling appeared to alter the macroscopic structure of *L. glaciale*.

Metamorphosis in *Lithothamnion*-conditioned filtered seawater was not significantly different from a filtered seawater control (Mann-Whitney U-test, $P > 0.05$), indicating that inducers are not leaking into surrounding seawater (Fig. 3). Thus, metamorphosis of urchin larvae in response to *L. glaciale* appears to require contact with the alga. The larvae are probably not responding to a diffusion gradient of inducer about live *L. glaciale*, since mild agitation

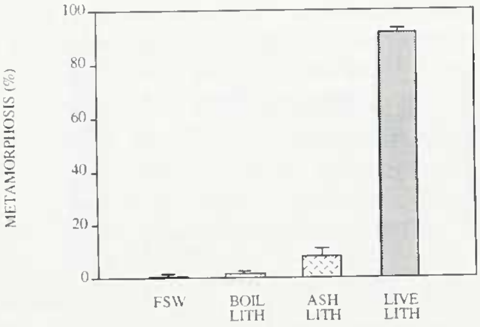

Figure 2. Mean percentage of *Strongylocentrotus droebachiensis* larvae that metamorphosed in response to filtered seawater (FSW), boiled *Lithothamnion glaciale* (BOIL LITH), ashed *L. glaciale* (ASH LITH), and live *L. glaciale* (LIVE LITH). Each treatment consists of 5 replicates with 25 larvae per replicate. Error bars indicate standard error.

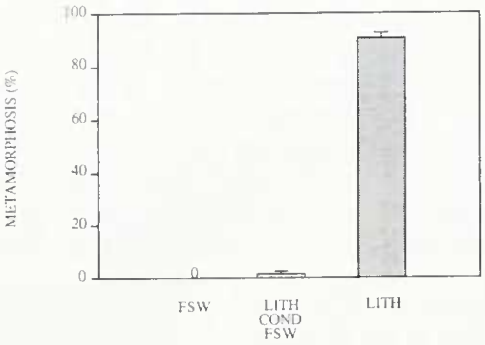

Figure 3. Mean percentage of *Strongylocentrotus droebachiensis* larvae that metamorphosed in response to filtered seawater (FSW), filtered seawater conditioned with live *Lithothamnion glaciale* (LITH COND FSW), and *L. glaciale* (LITH). Each treatment consists of 5 replicates with 25 larvae per replicate. Error bars indicate standard error. 0 denotes that no metamorphosed individuals were found.

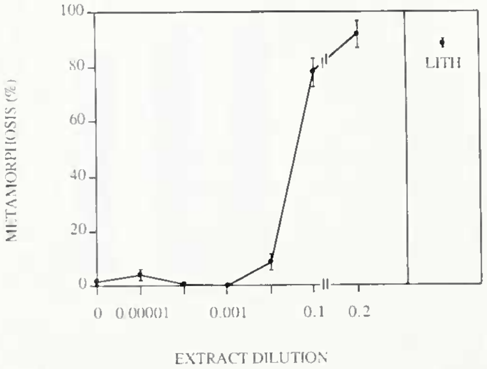

Figure 5. Mean percentage of *Strongylocentrotus droebachiensis* larvae that metamorphosed in response to serial dilutions of an extract of *Lithothamnion glaciale* and to intact *L. glaciale* (LITH). Each treatment consists of 5 replicates with 25 larvae per replicate. Error bars indicate standard error.

(which would disrupt any such gradient) did not reduce the rate of metamorphosis with *L. glaciale* (Mann-Whitney U-test, $P > 0.05$) (Fig. 4). This result provides further evidence of contact dependence.

Induction of larval metamorphosis in *S. droebachiensis* by a crude extract of *L. glaciale* in filtered seawater was concentration-dependent (Fig. 5); high rates of metamorphosis occurred at 1:5 (92%) and 1:10 (78%) dilutions, and these rates did not differ significantly from that with intact *L. glaciale* (88%) (Mann-Whitney U-test, $P > 0.05$ for both comparisons). Metamorphosis was minimal (<9%) at higher dilutions. The protein concentration of the algal extract within the range of effectiveness was between $\sim$30 μg ml^{-1} (1:10 dilution) and 60 μg ml^{-1} (1:5 dilution).

Induction by GABA also was concentration-dependent (Fig. 6). GABA induced larval metamorphosis at concentrations $\geq 10^{-5}$ *M*; the weakest concentration of GABA that induced metamorphosis in a proportion of larvae similar to that of a coralline algal control (*Phymatolithon*) was 10^{-3} *M* (Dunnett's test, $P > 0.05$). About 20 juveniles that metamorphosed in response to GABA were placed in running seawater and observed for a period of about 2 weeks. They appeared normal and active during this time.

Larvae metamorphosed in response to spores of *L. glaciale* adhering to the glass bottom of jars. The rate of metamorphosis in a treatment with spores was significantly higher than that in a filtered seawater control (Mann-Whitney U-test, $P < 0.05$), but significantly lower than with live *L. glaciale* (Mann-Whitney U-test, $P < 0.01$) (Fig. 7). This latter result may be explained by the surface area covered by spores which was only a small fraction of that covered by the alga (spores: <1 mm^2; *L. glaciale*: >900 mm^2). Settlement and metamorphosis in response to spores may have accounted for some of the

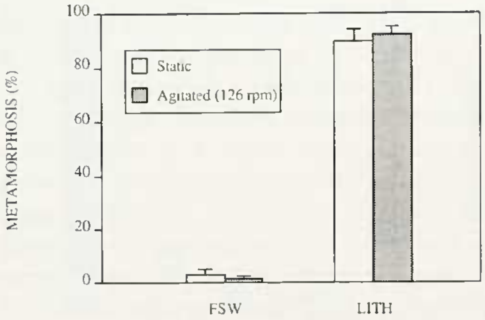

Figure 4. Mean percentage of *Strongylocentrotus droebachiensis* larvae that metamorphosed in response to filtered seawater (FSW) and *Lithothamnion glaciale* (LITH) under static (light bars) and agitated (dark bars) conditions. Each treatment consists of 5 replicates with 25 larvae per replicate. Error bars indicate standard error.

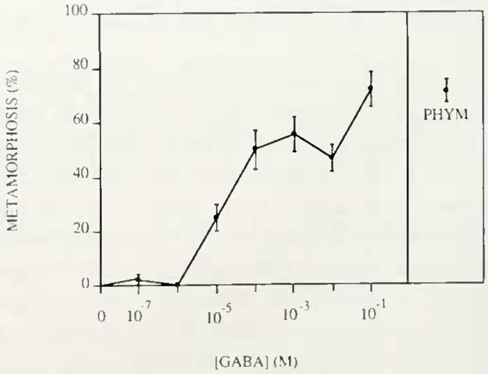

Figure 6. Mean percentage of *Strongylocentrotus droebachiensis* larvae that metamorphosed in response to various concentrations of γ-aminobutyric acid (GABA) and a *Phymatolithon* control (PHYM). Each treatment consists of 5 replicates with 10 larvae per replicate. Error bars indicate standard error.

recently metamorphosed individuals found on the bottom and sides of experimental jars after treatment with live *L. glaciale*. In treatments with live *L. glaciale* (pooled from 21 experiments), 24.5 ± 3.2% (mean ± SE) of all individuals were juveniles located on the bottom or sides of jars, whereas 60.9 ± 3.6% (mean ± SE) were juveniles on the alga.

Treating *L. glaciale* with antibiotics did not affect the rate of metamorphosis of *S. droebachiensis* (Fig. 8), although live bacterial numbers were significantly reduced with antibiotics (mean ± SD, treated: $5.60 \times 10^2 \pm 3.68 \times 10^1$ bacteria cm^{-2}, untreated: $9.86 \times 10^4 \pm 3.65 \times 10^4$ bacteria cm^{-2}) (one-tailed t-test, $P < 0.005$).

Discussion

Under static laboratory conditions, *Strongylocentrotus droebachiensis* showed a high rate of metamorphosis in response to three different morphological types of coralline red algae: a finely branched erect form (*Corallina officinalis*), a rugose crust with short nubby branches (*Lithothamnion glaciale*), and a smooth crust (*Phymatolithon*). In the field, however, passive entrapment of larvae may result in higher settlement on the more structurally complex branched and rugose corallines than on relatively smooth crusts. Dense aggregations of juveniles of *S. droebachiensis* (Scheibling, pers. obs.) and other small invertebrates (Keats *et al.*, 1984) have been observed on *C. officinalis* in the field. In eastern Newfoundland, Keats *et al.* (1984) found that juveniles of *S. droebachiensis* (2–6 mm test diameter) were most abundant on *L. glaciale* and rare on *Phymatolithon laevigatum*, *P. rugulosum*, and *Clathromorphum circumscriptum* (another smooth crust). However, the extent to which these observed distributions of juveniles in the field are determined by settlement processes or by differential mortality or migration is unknown. Flume experiments, examining settlement on algae with various morphologies, would be helpful in establishing the role of passive settlement in determining juvenile distribution patterns.

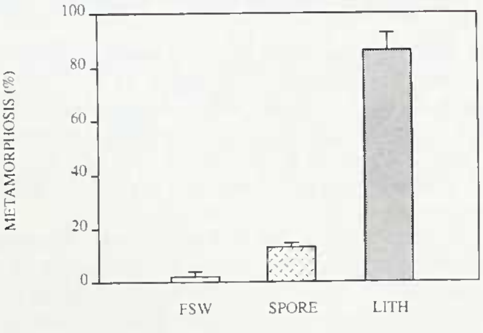

Figure 7. Mean percentage of *Strongylocentrotus droebachiensis* larvae that metamorphosed in response to filtered seawater (FSW), spores of *Lithothamnion glaciale* (SPORE), and *L. glaciale* (LITH). Each treatment consists of 5 replicates with 25 larvae per replicate. Error bars indicate standard error.

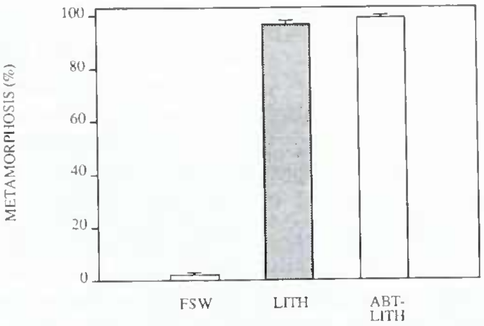

Figure 8. Mean percentage of *Strongylocentrotus droebachiensis* larvae that metamorphosed in response to filtered seawater (FSW), *Lithothamnion glaciale* (LITH), and *L. glaciale* treated with antibiotics (ABT-LITH). Each treatment consists of 5 replicates with 25 larvae per replicate. Error bars indicate standard error.

Metamorphosis of the larvae of *S. droebachiensis* appears to involve contact chemoreception. There is no evidence (from experiments with *Lithothamnion*-conditioned filtered seawater) that a chemical inducer is released into the water column (at least at concentrations that larvae can detect) or that larvae are responding to a diffusion gradient surrounding the alga. Boiling or ashing *L. glaciale* inactivates the inducing factor, suggesting that the inducer of metamorphosis of *S. droebachiensis* is a heat-labile molecule. Because these treatments kill algal tissues but do not visibly alter the surface contour of *L. glaciale* [the term contour is used to indicate that the scale of roughness is larger than the larva itself (Crisp, 1976)], thigmotaxis per se is probably not important in initiating metamorphosis.

Although induction of metamorphosis of *S. droebachiensis* may require contact with *L. glaciale*, recently metamorphosed individuals were not always located on the alga. Because larvae can be induced to metamorphose by isolated algal spores, some of these juveniles may have metamorphosed directly upon contact with spores released from *L. glaciale* onto the glass bottom of the jars. Alternatively, some larvae may land on the alga and receive a cue for metamorphosis, but then swim or crawl to adjacent areas before, or shortly after, metamorphosis. The latter phenomenon has been observed with the coral, *Agaricia tenuifolia*; the larvae require contact with the surface of crustose coralline algae to metamorphose, but subsequent attachment does not always occur directly on the algae (Morse *et al.*, 1988). In contrast, larvae of the abalone, *Haliotis rufescens*, settle and metamorphose exclusively on crustose coralline algae and not on adjacent non-algal surfaces (Morse *et al.*, 1980a).

Aqueous extracts of *L. glaciale* can induce metamorphosis of larvae of *S. droebachiensis*, indicating that grinding releases a water-soluble chemical cue. Larvae of the sea urchin, *Strongylocentrotus purpuratus* (Rowley, 1989), are induced to settle and metamorphose in response to the same small peptide inducer, purified from extracts of crustose coralline red algae (*Lithothamnium californicum*), that induces the larvae of *H. rufescens* to metamorphose (Morse *et al.*, 1984). These surface protein-linked oligopeptides have been demonstrated to be GABA-mimetic in their interaction with the larval receptors controlling metamorphosis of *H. rufescens* (Trapido-Rosenthal and Morse, 1986). GABA also triggers the metamorphosis of *S. droebachiensis*, but at higher concentrations (10^{-4}–10^{-3} M range) than those recorded for *H. rufescens* (10^{-6} M) (Morse *et al.*, 1980b).

A metamorphosis-inducing factor may be produced by coralline algae per se or by some component of the microbial film associated with these algae. Treating *L. glaciale* with antibiotics did not reduce the rate of metamorphosis of *S. droebachiensis*, even though the number of live bacteria on the surface of the alga was reduced by two orders of magnitude. However, some residual bacteria or other microbes (such as diatoms and protozoa) unaffected by antibiotics may be responsible for the production of an inducing factor.

Other laboratory studies of *Strongylocentrotus* spp. have shown that the larvae metamorphose in response to various substrata besides coralline red algae. Larvae of *S. purpuratus* showed similar rates of metamorphosis on rocks covered with coralline red algae and those with a marine microbial film and no coralline algae (Cameron and Schroeter, 1980). Rowley (1989) found that coralline red algae and red algal turf induced similar numbers of larvae of *S. purpuratus* to metamorphose, but that metamorphosis was significantly lower with filmed rocks. We have observed a high rate of metamorphosis of larvae of *S. droebachiensis* in response to a variety of macroalgae, including non-coralline brown, green, and red algae, as well as microbial and algal films (Pearce and Scheibling, in prep.). Thus, although adults of *S. droebachiensis* are frequently associated with coralline substrata, the factors triggering metamorphosis are apparently not specific to coralline red algae. This suggests that selective settlement of *S. droebachiensis* in coralline algal barren grounds rather than kelp beds may be less important than factors that limit larval supply to kelp beds [*e.g.*, deflection of water currents by kelp plants (Jackson and Winant, 1983)], larval predation by planktivorous fish (Tegner and Dayton, 1981; Gaines and Roughgarden, 1987) and suspension feeders (Pearse *et al.*, 1970; Bernstein and Jung, 1979)], or early post-settlement survival (Cameron and Schroeter, 1980; Harris *et al.*, 1984; Rowley, 1989).

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Associations Between Egg Capsule Morphology and Predation Among Populations of the Marine Gastropod, *Nucella emarginata*

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Abstract. Intraspecific variation in the morphology of egg capsules is ideal for assessing the costs and benefits of encapsulation, yet little is known about the extent of such variation among populations of a single species. In the present study, I compared capsule morphology among three populations of the intertidal gastropod, *Nucella emarginata*. Significant differences were found both in capsule wall thickness and capsule strength. Mean capsule wall thickness varied as much as 25% among populations, with the dry weight of capsular cases differing accordingly. Capsule strength, measured as resistance to puncturing and squeezing forces, also varied among populations, but did not directly reflect differences in capsule wall thickness. Despite extensive variation in capsule morphology within this species, the number and size of eggs contained within capsules of equal volume did not differ significantly among populations.

I also compared the type of capsule-eating predators that were present at each site. Shore crabs, *Hemigrapsus* spp., were abundant at all three sites; however, the predatory isopods *Idotea wosnesenskii* were only present at sites containing relatively thick-walled capsules. Although *Hemigrapsus* and *Idotea* were able to chew through both thick- and thin-walled capsules, laboratory experiments revealed that *Idotea* preferentially opened thin-walled capsules. These results suggest that variation in capsule morphology among populations of *N. emarginata* may, at least in part, reflect selection for the protection of embryos against predation.

Introduction

The confinement of developing embryos within elaborate egg capsules is a common phenomenon among marine invertebrates. Although this trait is widespread, few studies have addressed the benefits and costs associated with the production of encapsulating structures. Egg capsules may protect embryos from such environmental stresses as: predation (Pechenik, 1979; Perron, 1981), bacterial attack (Lord, 1986), osmotic changes (Pechenik, 1982, 1983; Hawkins and Hutchinson, 1988), desiccation (Spight, 1977; Pechenik, 1978), temperature shock (Spight, 1977; Pechenik, 1986), and wave action (Perron, 1981). Yet the ability of capsule walls to resist such stresses is known for only a few species (Emlen, 1966; Spight, 1977; Pechenik, 1978; 1982; 1983; Brenchley, 1982; Lord, 1986; Hawkins and Hutchinson, 1988), and only limited data are available on the survivorship of encapsulated embryos in the field (Spight, 1977; Pechenik, 1978; Brenchley, 1982). The production of egg capsules must also have associated costs. Capsule walls can divert a substantial amount of energy away from the production of eggs (Perron, 1981) and may also limit the availability of oxygen and nutrients to encapsulated embryos (Strathmann and Chaffee, 1984). If the adaptive significance of encapsulation is to be understood, these benefits and costs must be assessed.

Encapsulation of developing embryos is widespread among the more advanced gastropods (Pechenik, 1986). Neogastropod mollusks enclose embryos within structurally complex proteinaceous capsules and attach these structures to firm substrata in the marine environment. Within this group, egg capsule morphology varies tremendously (*e.g.*, Ostergaard, 1950; D'Asaro, 1970, 1988; Perron, 1981). Subtle differences in the properties of these

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capsules may reflect tradeoffs between benefits and costs of encapsulation (Perron, 1981; Perron and Corpuz, 1982). For instance, Perron (1981) found that capsule wall strength, and the proportion of reproductive energy invested in capsular cases, varied among closely related species of *Conus*. These differences were directly related to the development time of encapsulated embryos, such that embryos with long-term development were enclosed in thicker, stronger, and energetically more expensive capsules than those with short-term development (Perron, 1981; Perron and Corpuz, 1982). Thus, energetic costs associated with the production of strong capsular cases may be compensated for by the benefits of increased embryonic protection in species with protracted intracapsular development.

If variation in the morphology of egg capsules does reflect tradeoffs associated with specific benefits and costs of encapsulation, then intraspecific variation in capsular structure offers an ideal opportunity to assess such benefits and costs. Unlike interspecific comparisons, which may be subject to potentially confounding phylogenetic effects, studies of variation within a species can determine the importance of (1) physical constraints in female body size, (2) phenotypic responses to variation in environmental conditions and, (3) genetic divergence resulting from selection, in accounting for differences in capsule morphology. In no studies, however, have the structures of egg capsules in different populations of a single species been compared, and little is known about the extent of intraspecific variation in capsule morphology.

The widespread geographic distribution and direct development of the marine intertidal snail *Nucella emarginata* (Deshayes, 1839) (Prosobranchia: Muricidae) make this species an ideal candidate for studies of intraspecific variation in capsule morphology. These snails are common inhabitants of rocky shores from California to Alaska and range across wide extremes in wave exposure. *N. emarginata* deposit eggs year-round within 6–10 mm-long vase-shaped capsules and attach these structures directly to the substratum. After approximately 80 days of encapsulation (Emlen, 1966), embryos emerge as juvenile snails. Due to the absence of a planktonic larval stage in this species, gene flow among geographically separated habitats may be low (Palmer, 1984). As a consequence, snail populations may have become adapted to localized environmental conditions.

In this study, I examined variation in capsule morphology among three populations of *N. emarginata* separated along a gradient of wave-exposure. I compared capsule size, protective quality (as determined by wall thickness and capsule strength measurements), and capsule contents, among these study populations. Intraspecific differences in capsule morphology were examined with respect to site differences in the presence and abundance of capsule-eating predators. Previous studies, such as those by Emlen (1966) and Spight (1977), have indicated that predation on encapsulated *Nucella* embryos can be severe.

Materials and Methods

Study sites

This study was conducted at the Bamfield Marine Station on the west coast of Vancouver Island, British Columbia. Three study sites were established in Barkley Sound along a gradient of wave-exposure from sheltered to exposed: Grappler Inlet (48°49′N, 125°07′W), Ross Islets (48°52′N, 125°09′W), and Seppings Island (48°50′N, 125°12′W). Although no empirical studies have ranked these areas with respect to wave-exposure, my rating system, based on visual observations, corresponded with exposure scales used by others in the same geographic region (Austin *et al.*, 1971; Kitching, 1976; Craik, 1980; Crothers, 1984).

Intraspecific variation in capsule morphology

Snail size and capsule size. To compare the size of capsules produced by snails from each site, I collected 100 snails from each study area in March 1988. Collections were made by removing all living *Nucella emarginata* individuals from a given area, except for snails smaller than 10 mm in length, which were considered to be reproductively immature. Snails were brought into the laboratory, measured for shell length (apex to tip of siphonal canal) with Vernier calipers, tagged for identification, and then placed in mesh-panelled plastic containers (32 × 26 × 12 cm). Approximately 40–50 snails were held in each container and provided with barnacles (*Balanus glandula*) for food. Containers were kept submerged in seawater tanks and supplied with a continuous flow of fresh seawater. A snail was recognized to be laying an egg capsule only if it was found molding a new capsule with its ventral pedal gland. Other capsules were considered to be part of the same clutch if they were laid within a few millimeters of the freshly spawned capsule and were similar in shape and orientation (see Gallardo, 1979). Five capsules were collected from each female, and then preserved in 5% formalin (in seawater) for subsequent measurement. Egg capsule proteins are known to be stable in this fixative (Hunt, 1971).

I recorded the total capsule length, chamber length, and chamber width for each egg capsule. Total capsule length was the length of the capsule including the plug, but excluding the stalk, as the stalk length was known to be highly variable (Spight and Emlen, 1976). Chamber length and chamber width were measures of the maximum dimensions of the region housing developing embryos and nutritive nurse eggs. The volume of the capsule chamber was estimated from these measures using the formula for a prolate ellipsoid, $V = 4/3\pi(a/2)(b/2)^2$, where a = chamber length and b = chamber width (Pechenik, 1982).

Micromorphology of N. emarginata *egg capsules.* To examine the microstructure of *N. emarginata* capsules from each site, representative capsules were collected from field populations and then sectioned (unfixed) along sagittal and transverse planes using a freeze microtome. The wall microstructure of these sections was viewed under a compound light microscope and interpreted with reference to previous histological studies of the egg capsules of *N. lapillus* and other muricids (Bayne, 1968; Tamarin and Carriker, 1967; Sullivan and Maugel, 1984; D'Asaro, 1988).

The thickness of capsule walls was examined by taking serial cross-sections down the length of the chamber. Capsules were always selected from separate clutches to ensure that at least some were laid by different snails. Representative capsules were then measured, emptied of all contents by removing the capsular plug, and individually frozen on a freeze microtome. One section (10–12 μm thick) was taken at 10 percentile intervals along the length of the capsule chamber, starting at the opening of the plug region into the capsule chamber (0%) and ending at the base of the capsule chamber (100%). Sections were mounted in a seawater-soluble medium and then measured using a compound microscope with a calibrated ocular micrometer. Eight measurements of wall thickness were taken at approximately equal intervals around the circumference of each capsule section. The average wall thickness within each section was used in all subsequent data analyses.

To examine wall thickness differences among a large number of egg capsules, I established a laboratory population of 60 snails (30 males; 30 females) from each site. For each population, five male and five female snails were allocated to one of six replicate mesh-panelled plastic containers (26 × 16.5 × 13 cm), and were maintained in the laboratory as described above. Every two weeks, freshly laid capsules were collected from each container and placed in small mesh-panelled vials. Vials were then labelled, dated, and immersed in flowing seawater. Capsules required for experiments were selected by removing an equal number (whenever possible) from each replicate vial. Only relatively fresh capsules (within 4-weeks after deposition) were used in the following experiments, since capsule wall properties may change with age (see Roller and Stickle, 1988).

To examine variation in capsule wall thickness within and among populations of *N. emarginata*, 30 capsules were selected from each laboratory population. Each capsule was marked at a point 70% along the length of the capsule chamber. Preliminary data on wall thickness variation within a capsule indicated that capsule walls were thinnest and least variable in this region. For subsequent measurement, capsules were frozen on a freeze microtome and then sectioned at the marked region.

In addition, I examined variation in capsule wall thickness within and among clutches to determine whether females from the same population produced capsules of a similar wall thickness. For each laboratory population, egg capsules were collected from five different females by removing one clutch of capsules from each replicate container. Depending on clutch size, four to six capsules were selected from each clutch and then sectioned as described above.

To determine whether intraspecific variation in capsule wall thickness resulted in differences in the total amount of material allocated to capsular cases, I compared the dry weights of capsular cases from each site. Representative capsules were collected from each laboratory population, measured, and emptied of all contents. Stalks were removed from capsules to minimize variability in weight among capsules. Capsules were rinsed twice in distilled water, dried for 48 h at 75°C, and then weighed to 0.01 mg.

Capsule wall strength. I used two indices to measure the strength of egg capsule walls. The first index determined the resistance of capsule walls to puncturing forces and was based on Perron's (1981) procedure for *Conus* egg capsules. Freshly laid capsules were collected from laboratory populations and marked at a point 70% along the length of the capsule chamber. Capsules were then bisected by cutting along the two seams of the capsule chamber. Each capsule half was mounted individually between two pieces of Plexiglas (8.5 × 5 cm) and orientated such that a 1 mm diameter hole in each piece of Plexiglas was positioned directly over the marked region of the capsule chamber. A blunt-ended needle (0.36 mm^2 area), mounted beneath a flat weighing pan, was positioned over the Plexiglas such that the needle was perpendicular to the exposed capsule wall. Five-gram weights were sequentially loaded onto the weighing pan until the needle punctured the capsule wall. Each capsule half was punctured once. The mean puncturing force per capsule was used in all subsequent data analyses.

The second index of capsule strength measured the force needed to squeeze the plug out of intact capsules. Shore crabs (*Hemigrapsus* spp.) often ruptured *N. emarginata* egg capsules in this way by squeezing them in their chelae. Individual capsules were glued to a metal plate, which was then bolted to a vertical piece of Plexiglas mounted with strain gauges. A second metal plate was attached to a spindle so that this plate could be hand-cranked towards the mounted capsule. A chart recorder provided a record of the force required to rupture each egg capsule. This system was calibrated with known weights.

Capsule contents. Egg capsule contents were examined to determine whether the number or size of eggs per capsule differed among populations with respect to differences in capsule structure. Eighteen freshly laid capsules were collected from each laboratory population. Each capsule was measured and then emptied of all contents. As it proved difficult to distinguish between early developing embryos and nurse eggs, no attempt was made to separate nurse eggs from embryos. Before egg counts were made, however, a few embryos from each capsule were examined to ensure that they had not advanced past the second veliger stage. At this stage, embryos are able to feed on nurse eggs (LeBoeuf, 1971; Lyons and Spight, 1973).

Egg size was also compared among sites. As egg size was relatively constant within a capsule, only five eggs were sampled from each capsule. Length and width were measured for each egg using a compound microscope equipped with an ocular micrometer. As eggs were off-round in shape, volume was estimated by using the above formula for a prolate ellipsoid.

Predation on N. emarginata *egg capsules*

Laboratory experiments. A variety of abundant intertidal organisms was collected from each field site to determine which species might prey on *N. emarginata* capsules (nemertineans: *Emplectonema gracile;* annelids: *Nereis vexillosa;* mollusks: *Mopalia* spp., *Littorina scutulata, Onchidella borealis, Searlesia dira, Tegula funebralis, Nucella emarginata;* arthropods: *Pagurus granosimanus, P. hirsutiusculus, Hemigrapsus nudus, Hemigrapsus oregonensis, Idotea wosnesenskii, Gnorimosphaeroma oregonense, Cirolana harfordi;* echinoderms: *Leptasterias hexactis, Pisaster ochraceus;* chordates: *Oligocottus maculosus, Anoplarchus purpurescens*). Groups of individuals from each species were placed in appropriately sized mesh-panelled vials (3 × 3 × 6 cm) or containers (8 × 8 × 10 cm or 20 × 20 × 10 cm), and were provided with intertidal shells or bare rocks for shelter. Containers were partially immersed in seawater tanks and provided with a continuous flow of fresh seawater. Test animals were starved for 24 h before being presented with 8 intact *N. emarginata* egg capsules. Capsules were mounted on small flat rocks using a cyano-acrylate glue and arranged in a circular configuration. A predator was defined to have opened an egg capsule only if it ruptured or ate through the chamber containing developing embryos. Capsules were checked every 1–2 days for evidence of predation, and experiments were continued for at least two weeks or until all capsules had been opened. Five to ten replicates, including controls consisting of cages with no predators, were conducted for each species.

Field censuses of predation. In May 1988, two transects (10 m in length) were established parallel to the shoreline at Grappler Inlet (48°49′55″N; 125°07′03″W) at tidal heights of 1.2 and 2.2 m above extreme low water, spring [ELWS] (Canadian datum). Quadrats (0.25 m^2) were sampled at 0.5–1.0 m intervals along these transects to determine the abundance of *N. emarginata* and their egg capsules. Egg capsules were categorized on the basis of whether the capsule chambers were intact or ruptured. The age of intact capsules was estimated by noting the developmental stage of the embryos. New capsules were identified by the presence of nurse eggs, while older capsules contained well-developed shelled embryos. Ruptured capsules were also examined to determine whether they had been attacked by predators or whether developing embryos had hatched naturally. If capsules were empty, but had been chewed into the capsule chamber, they were considered to have been opened by predators. Such capsules were described by distinctive bite marks left on the capsule walls (see Fig. 9 below). The abundance of potential predators (identified from laboratory studies) was also censused along each transect.

In June 1988, three transects were established parallel to the rocky shoreline at the Ross Islets site (48°52′12″N, 125°09′36″W) at tidal heights of 1.9, 2.3 and, 2.6 m above ELWS. The two highest transects were positioned along a steeply sloping granite wall sparsely covered with *Fucus distichus, Balanus glandula,* and *Semibalanus cariosus.* The lowest transect was set along a boulder-covered beach directly below the higher transects. Data were collected as described above for the Grappler Inlet site. A large rocky outcropping (2.8–3.1 m above ELWS) adjacent to the study site was also censused in August 1988. This 16 m^2 area was divided into six equal-sized grids and a 0.25 m^2 quadrat was thrown haphazardly into each region. Snail density, egg capsule density, and predator abundance were recorded.

Censuses of snail density or predator abundance were not made at the Seppings Island site due to its extreme exposure to wave action. Capsule remains were regularly collected, however, to compare the type of predation among sites.

Susceptibility of capsules to predators

I also conducted a series of laboratory experiments to determine whether the intertidal isopods *Idotea wosnesenskii* could differentiate between thick- and thin-walled capsules. My field observations, and also those by Emlen (1966), indicated that these were important predators of *Nucella* egg capsules. As adult isopods were able to chew through all capsules regardless of wall thickness, I chose to compare the overall preferences of these predators for thick- and thin-walled capsules.

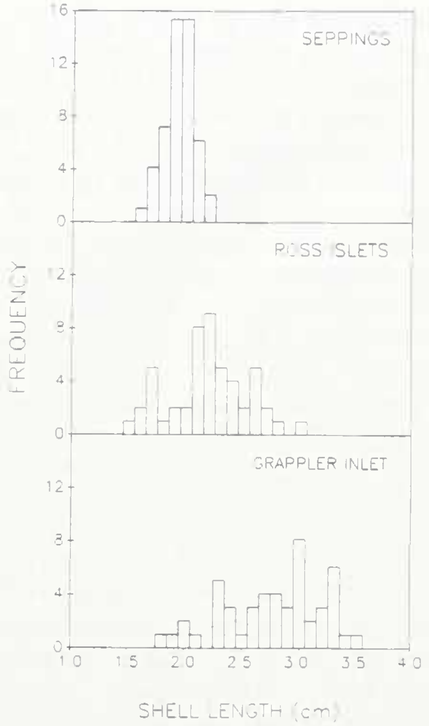

Figure 1. Size-frequency histograms of the first 50 *Nucella emarginata* individuals collected from each study site in March 1988. Snails smaller than 1.0 cm in shell-length are not included. Wave-exposure levels were predicted to be lowest at Grappler Inlet, highest at Seppings Island, and intermediate at Ross Islets. Mean shell lengths of snails are 1.9, 2.1, and 2.7 cm for Seppings, Ross Islets, and Grappler Inlet populations, respectively.

Adults of *Idotea* (mean length = 2.1 cm) were collected from Grappler Inlet in November 1988. Groups of three *Idotea* were placed in mesh-panelled cages (8 × 8 × 10 cm), and were then partially immersed in trays of fresh seawater. Predators were starved for an initial period of 24 h and then given five capsules from each of two snail populations (10 capsules in total). Predator preferences were tested for (1) thick *versus* thin-walled capsules (Grappler *vs.* Ross, and Seppings *vs.* Ross) and, (2) thick- *versus* thick-walled capsules (Grappler *vs.* Seppings). Capsules were arranged in a circular configuration, such that capsules from each population were interspersed. The number of capsules opened was recorded daily. Experiments were terminated when 4–6 out of 10 capsules had been opened. Five to ten replicate cages were used for each experimental combination.

Results

Intraspecific variation in capsule morphology

Snail size and capsule size. Snail size varied considerably among sites, with mean shell length increasing from wave-exposed to wave-sheltered shores (Fig. 1). Snail size at reproductive maturity also varied among populations. The smallest snails to spawn were 1.7, 2.1, and 2.7 cm in shell length from Seppings, Ross, and Grappler sites respectively, even though laboratory populations greatly overlapped in size (Seppings 1.4–2.2 cm; Ross: 1.4–3.0 cm; Grappler: 1.8–3.5 cm). Differences in the size of mature females within and among populations were reflected in the length of capsules produced (Fig. 2). Within each population, larger snails laid significantly longer capsules than smaller snails. Among populations, this trend was also apparent, although Seppings snails produced disproportionately large capsules per unit shell length (ANCOVA for slopes: $F = 1.20$, $P > 0.25$; ANCOVA for elevations: $F = 14.84$; $P < 0.001$). Hence, capsule size differed markedly among sites.

Micromorphology of N. emarginata *egg capsules.* Capsule walls of *N. emarginata* were composed of three laminae (L_1, L_2, and L_3; Fig. 3A,B) and were similar in structure to the capsule walls of other muricids (Sullivan and Maugel, 1984; D'Asaro, 1988). All measurements of capsule wall thickness were taken from the thick middle lamina (L_2), which consisted of a dense, fibrous middle layer (L_{2b}), sandwiched between two transparent, homogeneous layers (L_{2a} and L_{2c}). The outermost lamina (L_1) was extremely thin and often formed elaborate projections from the capsule wall. Consequently, this lamina was too difficult to measure reliably. The innermost capsule lamina (L_3) lined the capsule chamber and formed a transparent bag that enclosed developing embryos, nurse eggs, and intracapsular fluid. Sections in the apical region of the capsule indicated that this lamina was actually connected

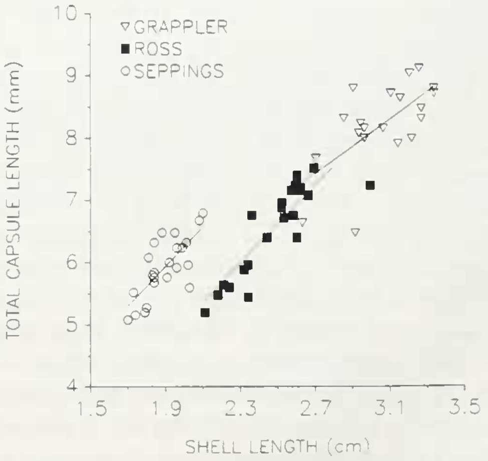

Figure 2. Relationship between total capsule length (excluding stalk) and shell length for laboratory-laid capsules from three populations of *Nucella emarginata*. Snails smaller than 1.7, 2.1, and 2.7 cm from Seppings, Ross Islets, and Grappler populations did not spawn. Least-squares linear regression equations for each site are: Seppings: $Y = 3.185X - 0.111$, $r^2 = 0.548$, $n = 23$; Ross Islets: $Y = 3.139X - 1.232$, $r^2 = 0.752$, $n = 23$; Grappler Inlet: $Y = 2.206X + 1.481$, $r^2 = 0.423$, $n = 20$.

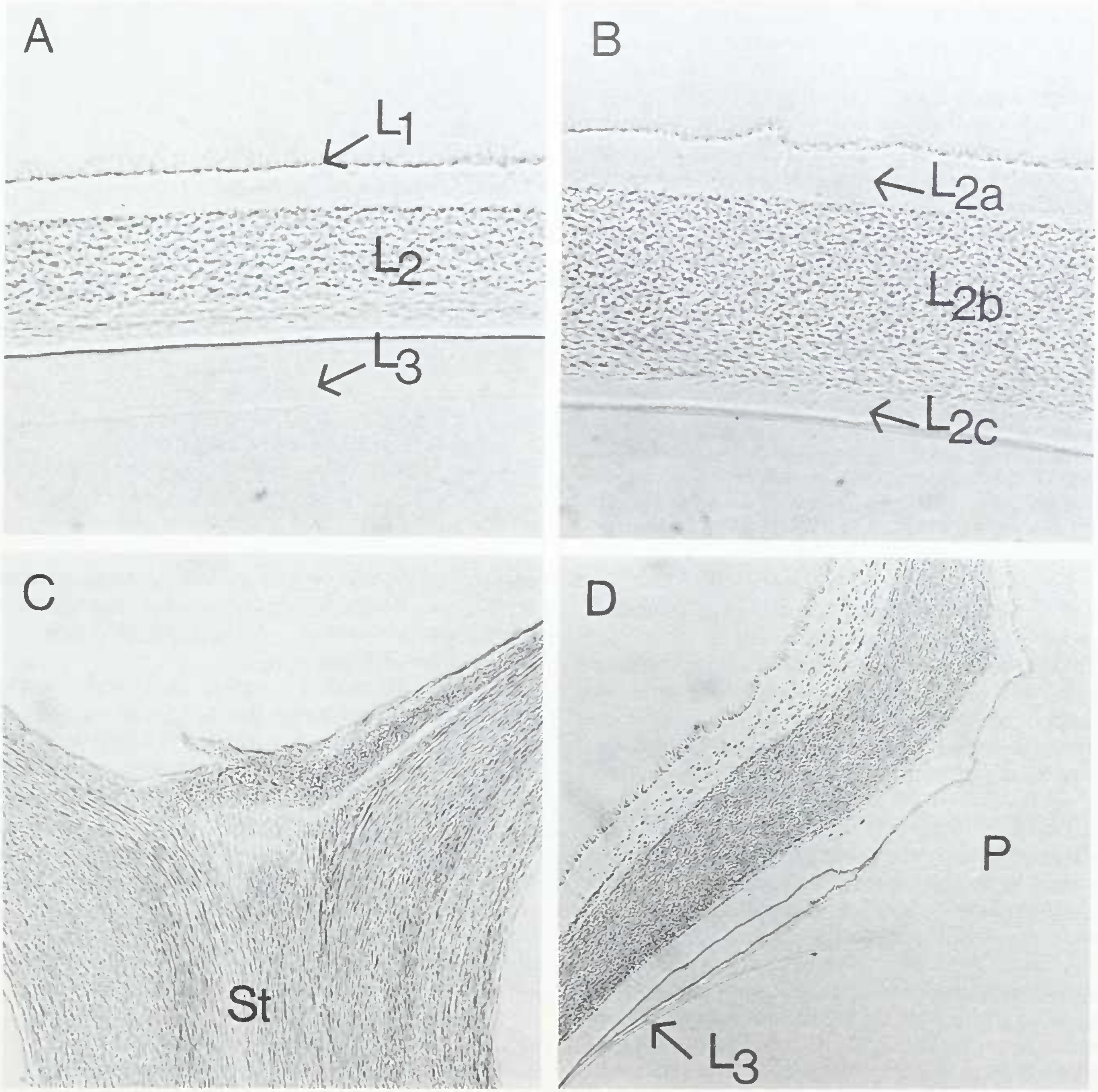

Figure 3. Microstructure of *Nucella emarginata* egg capsules: (A), (B) transverse sections taken 70% along the chamber of a capsule from Ross Islets (mean thickness = 60 μm) and Grappler Inlet (mean thickness = 90 μm), respectively; (C) longitudinal section through the capsule stalk; (D) longitudinal section through the capsule plug. Outer (L_1), middle (L_2), and inner (L_3) capsule wall laminae are indicated, as are the three component layers ($L_{2a,b,c}$) of the middle lamina, although note the disappearance of L_{2b} and L_{2c} in the vicinity of the stalk. The capsule plug (P) is also shown.

to the capsule plug and appeared to be composed of a similar material (Fig. 3D). The structure of the capsule wall was not homogeneous throughout the capsule, as is shown by longitudinal sections through the stalk and plug regions (Fig. 3C, D).

Serial sections along the chamber revealed considerable variation in capsule wall thickness (Fig. 4). Walls tended to be thickest in the plug and stalk regions and thinnest at a position 75% along the capsule chamber. Although capsule width also varied along length of the capsule

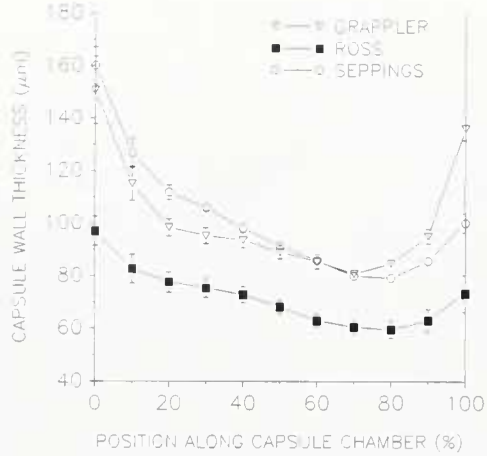

Figure 4. Variation in wall thickness along the capsule chamber of field-collected *Nucella emarginata* capsules from Grappler Inlet, Ross Islets, and Seppings Island. Serial sections were taken at 10% intervals along the capsule chamber, starting at the plug (0%) and ending at the stalk (100%). Values are expressed as mean ± 1 S.E. for 8 capsules sectioned from each population.

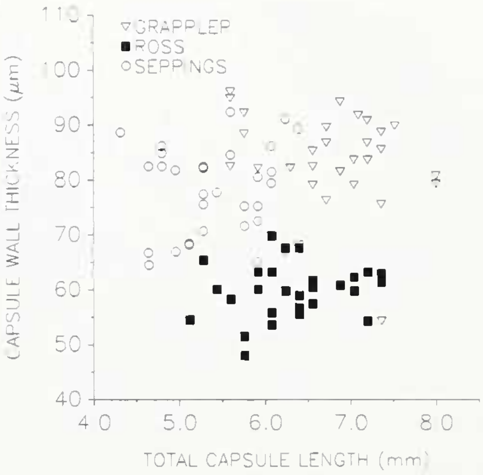

Figure 5. Variation in capsule wall thickness with total capsule length (excluding stalk) for 30 *Nucella emarginata* capsules from each laboratory population. Each value represents a mean of eight measurements taken from one section at a point 70% along the capsule chamber.

chamber, there was no correlation between capsule width and wall thickness (see Rawlings, 1989).

Capsule wall thickness also varied among populations (Fig. 4). At the 70th percentile division along the capsule chamber, Ross Islets capsules were significantly thinner than capsules from Seppings and Grappler (means of 61, 80 and 81 μm, respectively; ANOVA: F = 29.9, $P < 0.001$; Fig. 3A,B). This trend in wall thickness was apparent throughout the length of the capsule chamber. Differences in capsule wall thickness among populations resulted from variation in the thickness of all three component layers of the middle lamina (*i.e.*, $L_{2a,b,c}$), rather than in one component alone (data not shown).

Significant differences in capsule wall structure were also evident among laboratory-laid capsules (mean = 60, 78, and 83 μm, for Ross, Seppings, and Grappler capsules, respectively; ANOVA; F = 81.32, $P < 0.001$; Fig. 5). The wall thickness of these capsules did not differ significantly from capsules previously collected in the field (ANOVA; Grappler: F = 0.54, $P = 0.47$; Ross: F = 0.09, $P = 0.77$; Seppings F = 0.29, $P = 0.60$). Long-term exposure to the laboratory environment did not affect the morphology of capsules laid by these snails. Even after five months, snails still continued to produce their respective thick- or thin-walled capsules (data not shown).

Although the size of capsules varied extensively within and among snail populations, no relationship was evident between wall thickness and total capsule length (Fig. 5). Because capsule length was related to female shell length (Fig. 2), differences in capsule wall thickness within each population were probably not related to female size. Also, differences in capsule wall thickness among sites did not appear to reflect differences in snail size, as small Seppings snails (mean shell length = 1.9 cm) and large Grappler snails (mean shell length = 2.7 cm) both produced relatively thick-walled capsules.

Capsule wall thickness varied significantly among clutches within each population (ANOVA; Grappler, F = 3.44, $P = 0.02$; Ross, F = 32.05, $P < 0.001$; Seppings, F = 47.52, $P < 0.001$; Fig. 6). Variation in capsule wall thickness among clutches, however, did not obscure dif-

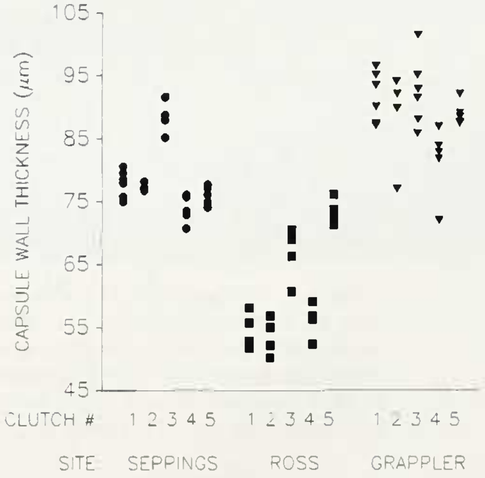

Figure 6. Variation in capsule wall thickness within and among clutches of *Nucella emarginata* capsules. Five clutches, each from a different female snail, were sampled from all three laboratory populations, with n = 6, n = 4, and n = 6 capsules/clutch for Seppings, Ross Islets, and Grappler populations, respectively. Each data point represents the mean of eight measurements taken from one section at a point 70% along the capsule chamber. Each vertical group of points represents one clutch of capsules.

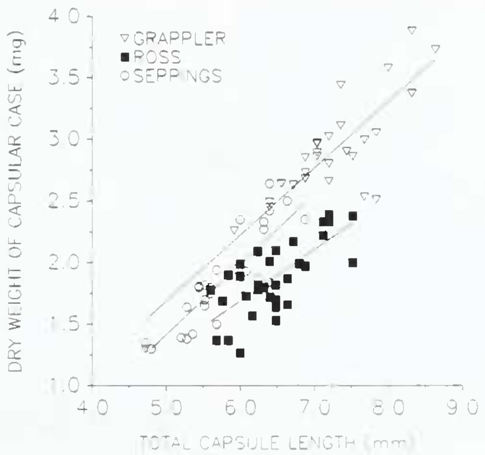

Figure 7. Dry weight of empty capsular cases as a function of total capsule length (excluding stalk) for each laboratory population. Each data point represents one capsule. Least-squares linear regression equations for each population are: Seppings: $Y = 56.859X - 142.901$, $r^2 = 0.701$, $n = 27$; Ross Islets, $Y = 41.359X - 79.242$, $r^2 = 0.523$, $n = 33$; Grappler Inlet, $Y = 53.873X - 100.746$, $r^2 = 0.740$, $n = 28$.

ferences in capsule wall thickness among sites. Ross Islets capsules ranged in wall thickness from 50 to 76 μm, while Seppings and Grappler capsules ranged from 71 to 92 μm and 72 to 102 μm, respectively.

Longer capsules had significantly heavier dry weights for each laboratory population of snails (Fig. 7). Although the slopes of these site-specific relationships were not significantly different (ANCOVA for slopes; $F = 1.20$, $P > 0.25$), the elevations did vary significantly (ANCOVA for elevations; $F = 50.52$; $P < 0.001$). These differences corresponded well with those in capsule wall thickness among populations, as thin-walled Ross Islets capsules weighed significantly less for a given length than thicker-walled capsules from the other two sites. Grappler capsules were also significantly heavier than Seppings capsules, again reflecting the differences reported above in wall thickness.

Capsule wall strength. The force required to puncture capsule walls differed among the three laboratory populations (Table I). Grappler capsules were significantly more resistant to puncturing than capsules from the other two sites (ANOVA, $F = 11.77$, $P < 0.001$; Tukey Multiple Comparison Test, $P < 0.05$). Ross Islets and Seppings capsules did not differ significantly in puncturing resistance (Tukey M.C.T., $P > 0.05$), despite the fact that Seppings capsules had substantially thicker walls (Fig. 5).

The force needed to rupture capsules by squeezing also varied among laboratory populations (Table I). Grappler capsules required significantly larger forces to rupture the capsular plug than did either Ross Islets or Seppings capsules (ANOVA, $F = 18.67$, $P < 0.001$; Tukey M.C.T., $P < 0.05$). Thick-walled Seppings capsules were also slightly more resistant to squeezing than thin-walled Ross Islet capsules, however, this difference was not significant (Tukey M.C.T., $P > 0.05$).

Capsule contents. No significant differences were observed in the total number of eggs allocated to Seppings, Ross Islets, and Grappler capsules (Fig. 8). Neither the slopes (ANCOVA for slopes: $F = 0.65$, $P > 0.50$) nor elevations (ANCOVA for elevations: $F = 0.36$; $P > 0.50$) of these relationships differed significantly among populations.

The size of *N. emarginata* eggs was also relatively constant. Although Ross Islets capsules contained slightly larger eggs than Grappler or Seppings capsules (mean egg volume = 40.5×10^{-4} mm^3, 39.6×10^{-4} mm^3, and 38.1×10^{-4} mm^3, respectively), these differences were not significant (ANOVA: $F = 1.55$, $P > 0.22$).

Predation on N. emarginata *egg capsules*

Laboratory-identified predators. Only three types of invertebrates opened *Nucella emarginata* egg capsules in the laboratory: isopods (*Idotea wosnesenskii*), shore crabs (*Hemigrapsus nudus* and *H. oregonensis*), and chitons (*Mopalia* spp.).

Idotea wosnesenskii regularly preyed upon egg capsules in laboratory experiments. These predators usually opened capsules by chewing through the side of the capsule chamber and left bite-marks as shown in Figure 9 (A, E).

Table I

Intraspecific variation in the wall thickness and strength of Nucella emarginata *capsules*

	Mean ± S.E.[a]					
	Grappler Inlet	(n)	Seppings island	(n)	Ross Islets	(n)[b]
Capsule wall thickness (μm)	83.3 ± 1.6	(30)	78.4 ± 1.4	(30)	59.9 ± 0.9	(30)
Puncturing force (MN/m^2)	6.18 ± 0.20	(10)	4.92 ± 0.14	(10)	5.17 ± 0.17	(15)
Popping force (N)	14.5 ± 1.0	(19)	9.7 ± 1.0	(15)	7.5 ± 0.6	(21)

[a] For each index, populations not connected by a horizontal line are significantly different from one another (Tukey M.C.T. at $\alpha = 0.05$).

[b] (n) refers to the number of capsules sampled from each population.

Caged *Idotea* (1.6–3.2 cm in body length; mean = 2.2 cm) opened a mean (± S.E.) of 4.6 ± 0.8 capsules over a five-day period (n = 11). Predation rates varied among these individuals, but not in relation to size or sex, although newly hatched *Idotea* (5 mm in length) did not open egg capsules in the laboratory. Two other species of intertidal isopods, *Gnorimosphaeroma oregonense* (mean length = 0.9 cm) and *Cirolana harfordi* (mean length = 1.4 cm), nibbled capsules extensively, but never chewed through capsule walls.

The shore crabs *Hemigrapsus nudus* and *H. oregonensis* also readily opened *N. emarginata* capsules in the laboratory. Predation rate was dependent on crab size. Small and medium-sized *H. nudus* (carapace widths of <1.5 cm and 1.5–2.5 cm, respectively) opened a mean (± S.E.) of 3.3 ± 0.8 (n = 10) and 6.0 ± 0.7 (n = 18) capsules respectively over a 5-day period. Larger crabs (carapace width > 2.5 cm) opened all eight capsules after only 3 days (n = 9). *Hemigrapsus* spp. exhibited two methods of opening *Nucella* capsules. Larger crabs typically ruptured the capsular plug by squeezing the capsule chamber in their chelae. More often, however, crabs tended to chew through the plug region directly into the capsule chamber, as shown in Figure 9(B, F).

Few *N. emarginata* capsules were opened by *Mopalia* spp. in laboratory tests. Over a 2-week period, 21 chitons only opened 6 of 56 capsules. These predators usually rasped capsules open near the base of the chamber (Fig. 9C, G), and sometimes completely severed the capsule chamber from the stalk.

Field censuses of predation. Snails and egg capsules were most abundant in the lower regions of the intertidal channel at Grappler Inlet (Table II), with egg capsules being deposited deep within a dense meshwork of mussels and barnacles. Egg capsule predators *Hemigrapsus oregonensis* (1.0–2.2 cm in carapace width), *Idotea wosnesenskii* (2.1–2.9 cm in body length) and *Mopalia* spp. (2–6 cm in body length), were present in this region, with *Mopalia* spp. being the most numerous. Eighteen percent of capsules collected along this lower transect had been opened by predators (Table II). Predators of many of these capsules could be identified by distinctive bite marks left on capsule walls (Fig. 9). The majority of capsules showed evidence of predation by *Idotea*, even though these isopods were scarce at the time of censusing. Capsules collected from three intertidal boulders showed similar types of predation, with the percentage of capsules opened by predators ranging from 11 to 26% (mean = 18%). Although there was no direct evidence of predation by *Hemigrapsus* spp. at this site, these crabs may have been responsible for opening many torn and chewed capsules whose bite marks could not be readily identified. Some capsules were also emptied by means of bevelled holes (0.4 × 0.2 mm; Fig. 9D, H). Predators of these capsules may have been intertidal gastropods, because they have been reported to make similar holes in other gastropod egg capsules (Abe, 1983).

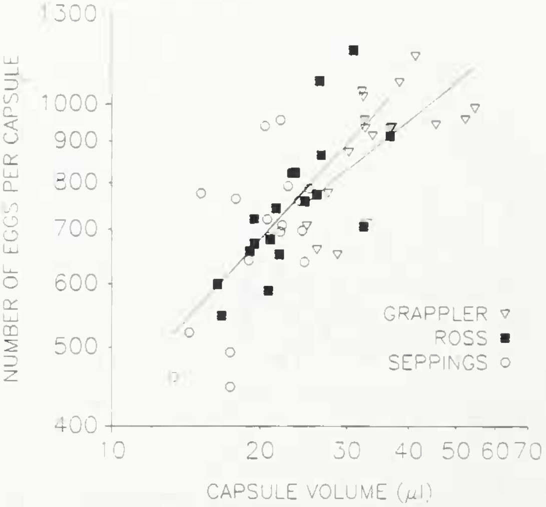

Figure 8. Relationship between the number of eggs per capsule and the volume of the capsule chamber for *Nucella emarginata* individuals from Seppings, Ross Islets, and Grappler Inlet. Counts of eggs include both developing embryos and non-developing nurse eggs. Least-squares linear regression equations for each population are: Seppings: $\text{Log } Y = 0.676 \text{ Log } X + 1.954$, $r^2 = 0.551$, $n = 18$; Ross Islets: $\text{Log } Y = 0.669 \text{ Log } X + 1.962$, $r^2 = 0.340$, $n = 18$; Grappler: $\text{Log } Y = 0.492 \text{ Log } X + 2.192$, $r^2 = 0.410$, $n = 18$.

The density of snails and egg capsules was lower along the high transect at Grappler Inlet (Table II). In contrast, *Hemigrapsus* and *Idotea*, were notably more abundant, and the percentage of capsules opened was also higher, with 32% of capsules showing evidence of predation. *Idotea* bite-marks were found on all capsular remains.

Densities of snails and egg capsules varied markedly among transects at the Ross Islets site (Table II). All capsules at this site were attached to vertical surfaces or overhangs. Encapsulated embryos were also generally further developed than those at Grappler Inlet, reflecting the fact that censuses were made approximately a month later. *Hemigrapsus nudus* (0.6–2.4 cm in carapace width) were the only known predators of *Nucella* egg capsules at this site, with densities ranging up to 360/m^2. The majority of opened egg capsules also appeared to have been preyed upon by *Hemigrapsus* (Table II).

Egg capsules from Seppings Island showed evidence of bite-marks by both *Idotea* and *Hemigrapsus*. Despite the extreme levels of wave action at this site, these predators were abundant, especially within the thick beds of *Mytilus californianus*. Capsules were also found with bevelled holes identical to those collected from Grappler Inlet (Fig. 9D, H).

Susceptibility of capsules to predators

Idotea opened thin-walled capsules from Ross Islets more frequently than thick-walled capsules from either

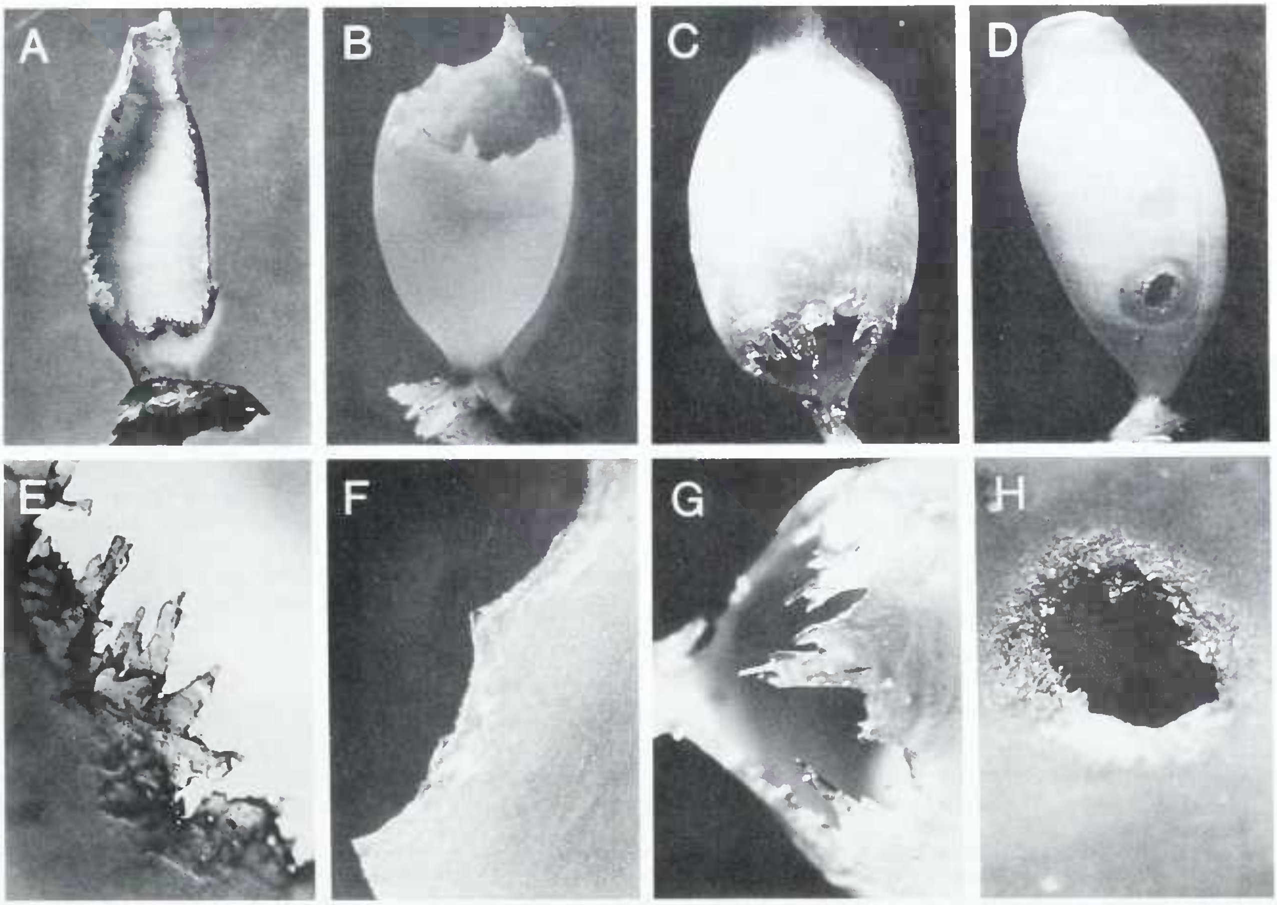

Figure 9. Characteristics of species-specific predation on *N. emarginata* egg capsules by: *Idotea wosnesenskii* (A, E), *Hemigrapsus* spp. (B, F), *Mopalia* spp. (C, G), and an unknown predator (D, H), possibly an intertidal gastropod. For each type of predator, a whole mount of the opened capsule is shown (Mag: 8X), with a close-up below illustrating the characteristic bite-marks (Mag: 25-50X).

Grappler Inlet or Seppings Island (Table IIIA, B). In 10 trials, 35 Ross Islet (thin-walled) capsules were opened compared with 15 Grappler (thick-walled) capsules (Fisher's Exact Test, $P = 0.0001$). Ross Islets (thin-walled) capsules were also opened more frequently than Seppings (thick-walled) capsules, although this difference was not quite significant (16 Ross Islets capsules *versus* 10 Seppings capsules; Fisher's Test, $P = 0.08$). In contrast, isopods did not exhibit any preferences for Seppings *versus* Grappler capsules (10 Grappler *versus* 9 Seppings capsules; Fisher's Test, $P = 0.50$; Table IIIC), and in two of six trials no capsules were eaten during a ten-day period. Hence, thick-walled capsules from Grappler Inlet and Seppings Island were more resistant to predation than thin-walled capsules from Ross Islets.

Discussion

Intraspecific variation in capsule morphology

The morphology of *N. emarginata* capsules varies extensively among populations. In the present study, both capsule wall thickness and strength differed significantly among the three intertidal locations examined. Such variation in capsular structure may reflect (1) physical constraints associated with female body size, (2) phenotypic differences in response to variable environmental conditions, or (3) genetic divergence caused by selection.

Intraspecific differences in the wall thickness and strength of *N. emarginata* capsules may reflect constraints associated with female size. Although the morphology of neogastropod egg capsules is governed by the size of the capsule gland, which, in turn, is restricted by female shell-length (Spight *et al.*, 1974; Spight and Emlen, 1976; Perron and Corpuz, 1982; present study), little is known about the direct effect of female size on capsule wall structure. Perron and Corpuz (1982) reported that wall thickness and strength of *Conus pennaceus* capsules increased with capsule size and snail shell-length. Their results suggested that the structure of capsule walls may be limited by the size of the capsule gland. In the present study, capsule size and snail shell-length varied markedly within and

Table II

Summary of Nucella emarginata *egg capsules, and potential egg capsule predators censused along transects at Grappler Inlet and Ross Islets study sites*

	Density[a] (Mean/m^2 ± S.E.)					Census of egg capsules[b]									
							Chamber intact		Chamber ruptured		Predators of opened capsules[c]				
	Nucella emarginata	Egg capsules	*Hemigrapsus* spp.	*Idotea* spp.	*Mopalia* spp.	# of capsules	Early embryos	Late embryos	Hatched naturally	Opened by predators	H	I	M	U.G.	UNID
Grappler Inlet															
Tidal height															
1.2 m															
(n = 8)	29.6 ± 8.8	126.0 ± 47.6	4.9 ± 0.5	0.4 ± 0.4	9.3 ± 2.1	255	35	22	22	18	4	43	2	17	34
						494[d]	24	50	0	18	3	82	0	1	14
2.0 m															
(n = 8)	14.0 ± 6.0	9.6 ± 5.2	10.2 ± 3.1	8.0 ± 3.7	0	19	68	0	0	32	0	100	0	0	0
Ross Islets															
Tidal height															
1.9 m															
(n = 6)	26.0 ± 10.4	24.8 ± 19.2	360.8 ± 38.6	0	0	37	46	5	24	24	100	0	0	0	0
2.3 m															
(n = 8)	6.4 ± 4.4	70.0 ± 30.4	0	0	0	140	19	13	45	22	77	0	0	0	23
2.6 m															
(n = 8)	289.6 ± 97.6	5.2 ± 4.8	0	0	0	10	10	0	0	0	0	0	0	0	0
2.8–3.1 m															
(n = 6)	203.3 ± 30.3	744.6 ± 17.2	24.6 ± 6.4	0	0	1117	0	7	70	22	73	0	0	0	26

[a] Censuses were made in May 1988 and June 1988 for Grappler Inlet and Ross Islet study sites, respectively. The number (n) of 0.25 m^2 quadrats used to estimate these densities is shown for each transect.

[b] Intact capsules were aged by examining the developmental stages of enclosed embryos. Empty capsules were categorized according to whether embryos had hatched naturally or had been opened by predators. Data are expressed as a percentage of the total number of capsules along each transect that were found in each category. Percentages may not always add up to 100%, because some capsules were found intact but their contents were dead.

[c] Predators responsible for opening capsules were identified by means of bite-marks left on the capsule chamber. Abbreviations: H = *Hemigrapsus* spp., I = *Idotea* spp., M = *Mopalia* spp., U.G. = unknown gastropod, and UNID = unidentified predators. Capsules in the "UNID" category had been opened by predators, but bite-marks could not be accurately identified. Data in each category represent a percentage of the total number of capsules opened by predators along each transect.

[d] These capsules were collected in Aug 1988 from three intertidal boulders in Grappler Inlet.

among the Grappler, Ross Islets, and Seppings populations. Capsule wall thickness, however, did not differ as predicted with either capsule length or snail shell-length. Hence, variation in the thickness of capsule walls among *N. emarginata* populations was not the result of allometric contraints associated with female size.

Differences in capsule structure among populations of *N. emarginata* also did not appear to be the result of phenotypic plasticity. Variation in capsule wall thickness within a clutch was low compared to variation among clutches produced by different individuals. Hence, within a spawning period, individual females deposited capsules of relatively consistent wall thickness. Also, snails continued to produce their respective thick- or thin-walled capsules even after five months in the laboratory, a period during which snails from all three populations were kept under similar environmental conditions. Thus, differences in the structure of egg capsules were not likely to be short-term phenotypic responses to site differences in diet, food abundance, or levels of environmental stress. Such results suggest that the production of thick or thin capsule walls may be an adaptive response to environmental conditions.

Costs associated with producing thick-walled capsules

There are likely to be both costs and benefits associated with the production of thick-walled capsules. Thick-walled capsules may incur a greater energetic cost than thin-walled capsules based on their greater dry weight per unit length. For instance, thin-walled capsular cases from the Ross Islets (6.5 mm in length) weighed 24% less than thick-walled capsules from Grappler Inlet, and 16% less than thick-walled capsules from Seppings Island. As capsular cases can account for more than 50% of the dry weight of intact capsules (*i.e.*, including the eggs; Roller and Stickle, 1988; Rawlings, unpub. data), and as *N. emarginata* capsular material has almost the same energy content per unit weight as the eggs (22.6 KJ per ash-free gram compared to 25.1 KJ per ash-free gram of embryos; J. Davis, unpub. class project, Friday Harbor Laboratories, 1984),

Table III

Preferences of Idotea wosnesenskii *for thick- or thin-walled egg capsules of* Nucella emarginata

Site Comparisons	Mean (±S.E.) number of capsules opened[a] Thin-walled	Thick-walled
A) Ross *vs.* Grappler	3.5 ± 0.3	—
(n = 10)	—	1.5 ± 0.3
B) Ross *vs.* Seppings	3.2 ± 0.4	—
(n = 5)	—	2.0 ± 0.5
C) Grappler *vs.* Seppings	—	2.5 ± 0.3
(n = 6)[b]	—	2.3 ± 0.3

[a] Average number of egg capsules opened by *I. wosnesenskii* when given a choice of capsules from two different study sites. Predators were placed in cages with 10 capsules (5 from each site), and the first 5 capsules to be opened were recorded. Data are expressed as the mean number (±1 S.E.) of capsules selected from each site, where (n) refers to the number of replicates performed for each comparison.

[b] In 2 out of 6 replicates, no capsules were eaten over a 10-day period.

the energy spent in producing thicker capsule walls must represent either a substantial decrease in the energy available for egg production or an increase in the reproductive effort of an individual. In fact, Perron (1982) found that the production of thick, puncture-resistant, capsule walls among *Conus* spp. was associated with a higher annual reproductive effort than the production of weak, thin-walled capsules. In the present study, I did not compare reproductive effort among populations. The production of thick-walled capsules, however, was not associated with a reduction in egg size or number of eggs contained per unit capsule volume. Hence, on a per capsule basis, there was no evidence of a tradeoff between the amount of energy invested in capsular cases versus eggs.

Other potential costs still remain to be tested. For instance, Strathmann and Chaffee (1984) have suggested that thick encapsulating structures may reduce the availability of nutrients and oxygen to developing embryos. Hence, (1) the density of embryos per capsule, (2) the developmental rate of embryos, or (3) the proportion of embryos surviving, may differ between thick- and thin-walled capsules. Although preliminary results have indicated that there are no significant differences between the number of embryos contained within thick- and thin-walled capsules (Rawlings, 1989), further comparisons still need to be made.

Benefits of enclosing eggs within thick-walled capsules

Numerous studies have examined interspecific differences in the properties of gastropod egg capsules (Perron, 1981; Perron and Corpuz, 1982; Pechenik, 1983, D'Asaro, 1988). The degree to which thick-walled capsules protect developing embryos better than thin-walled capsules, however, is still unclear. Pechenik (1983), for example, found that the rate of salt movement across the walls of *Nucella lamellosa, N. lapillus,* and *N. lima* capsules did not vary systematically with capsule wall thickness. Hence, the resistance of capsule walls to osmotic shock or desiccation stress might not differ between thick- or thin-walled structures. Such interspecific comparisons may be confounded by differences in the structural components of capsule walls, however, which vary considerably among *Nucella* species (pers. obs.).

The only previous evidence to support the hypothesis that strong, thick-walled capsules are more protective than weak, thin-walled capsules has come from positive correlations between capsule strength, the proportion of reproductive energy invested in capsule walls, and developmental time of encapsulated embryos among *Conus* species (Perron, 1981; Perron and Corpuz, 1982). Although capsule wall thickness was not compared among all species, *Conus pennaceus,* with encapsulated development times of 26 days, was found to have significantly thicker capsule walls than *Conus rattus,* with encapsulated development times of 11 days (Perron and Corpuz, 1982). These results indicate that strong, thick-walled capsules may reflect selection for increased protection of embryos when exposure to environmental stresses is long. As yet, however, no selective mechanism has been identified to explain this pattern.

Perron (1981) has suggested that egg capsule predators may be the agent of selection for strong, energetically expensive capsule walls. Indeed, predation appears to be an important source of mortality among encapsulated embryos. For instance, Brenchley (1982) found that 52% of the capsules of the mud snail, *Ilyanassa obsoleta,* were opened by crabs or snails during 10 days of a development period lasting up to 3 weeks. Spight (1972) noted that predators had opened 77% of *Nucella lamellosa* capsules in some spawning aggregations. Other studies, such as those by MacKenzie (1961), Haydock (1964), Emlen (1966), and Abe (1983), have also documented high levels of predation on gastropod egg capsules. In the present study, one-time field censuses of predation on *N. emarginata* egg capsules indicated that up to 32% of capsules had been opened by crabs, isopods, and other predators. Therefore, predators are responsible for considerable mortality among encapsulated embryos.

Thick-walled capsules may be more difficult to open or require longer handling times by predators than thin-walled capsules. Hence, the former might be selected for in areas where predators are abundant. The production of thick-walled *N. emarginata* egg capsules was not related to the relative abundance of *Hemigrapsus* spp. among

Grappler Inlet, Seppings, and Ross Islet study sites. In fact, thin-walled capsules were found at Ross Islets, where crabs densities reached up to 360/m^2. In contrast, the predatory isopod *Idotea wosenesenskii* was found only at the two sites where thick-walled capsules were present. Embryos contained within thick-walled capsules were also less likely to be eaten by *Idotea* than those contained within thin-walled capsules. Hence, these results indicate not only that thick capsule walls protect developing embryos better against *Idotea* than thin capsule walls, but also that these predators may have resulted in selection for thick-walled capsules at Grappler Inlet and Seppings Island study sites.

Although capsule wall thickness varied in accordance with the presence of *Idotea,* capsule strength did not. The fidelity with which puncture-resistance and squeezing forces—my measures of capsule strength—simulate methods used by *Idotea* to open capsules is not known. Possibly, however, these measures of capsule strength could reflect the action of other environmental stresses affecting encapsulated embryos. Desiccation (Feare, 1970; Spight, 1977; Pechenik, 1978), osmotic stress (Pechenik, 1982; 1983; Hawkins and Hutchinson, 1988), wave-action (Perron, 1981), bacterial attack (Lord, 1986), and thermal stress (Spight, 1977; Pechenik, 1986) are all potentially important sources of mortality for encapsulated embryos. These stresses may have independently resulted in the selection of different properties of capsule walls.

Confounding influences in intraspecific comparisons

Although intraspecific variation in capsule morphology may provide the best opportunity to address costs and benefits of encapsulation, interpretations of differences among populations may be confounded by the effects of environmental stresses on adult snails. Environmental stresses, such as wave-exposure, affect the reproductive effort of gastropods profoundly. For instance, wave-exposed snails typically mature at smaller sizes and exhibit higher reproductive efforts over shorter lifespans than longer-lived, wave-sheltered snails (Roberts and Hughes, 1980; Calow, 1981; Etter, 1989). Similarly, *N. emarginata* from Seppings matured at smaller sizes and produced proportionally larger capsules than those from Grappler Inlet (Fig. 2). How such differences in reproductive effort might be reflected in the partitioning of energy between eggs and extraembryonic products is unclear. Nevertheless, the type of capsule produced should still depend on the relation between energetic cost and the defensive effectiveness of capsular material.

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The Chromophore and Polypeptide Composition of *Aplysia* Ink

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Abstract. The composition of the ink of the sea hare, *Aplysia*, was studied in regard to its tetrapyrrole and polypeptide content. The ink was separated into three pigment components by both thin-layer and gel filtration chromatography. These three pigments have distinctive visible absorption spectra, and—by comparison with known tetrapyrroles—we have demonstrated that they are derived from the three tetrapyrrole chromophores (bilins) found on the biliproteins of certain red algae, which constitute a portion of the *Aplysia* diet. The red component is phycourobilin; the purple is phycoerythrobilin; and the blue is phycocyanobilin. Sodium dodecyl sulfate gel electrophoresis experiments were also performed. The results of these experiments showed several polypeptides, and major bands at 78,000 and 61,000 molecular weight were noted. Biliproteins, at most, would be minor components of the ink.

Introduction

Aplysia, the sea hare, is a marine mollusk whose principal habitats are the littoral and sublittoral regions, where it feeds on various seaweeds [see Kandel (1979) for a review]. *Aplysia* discharge a dark purple ink from a gland (called the ink, purple, or Bloehmann's gland) located on the edge of its mantle shelf. The ink has been viewed by some biologists as a defense against predators, but there is no consensus on how it would perform this function. Eales (1921) suggested that the ink is a screen used by the *Aplysia* to cloud its escape from danger. Others have disagreed, noting that an ink cloud might be effective in shallow, calm waters but not in sublittoral water, where it would be diluted too quickly to protect the slow-moving sea hare (Carew and Kandel, 1977). Others concluded that the ink would not be effective as a screen even in shallow water (Kupfermann and Carew, 1974; Linton, 1966). DiMatteo (1981, 1982) found that the ink alone, or when injected into food, is avoided by seagulls and crabs, who apparently find it distasteful or toxic. Nondefense functions, such as excretion or signalling, have also been attributed to the ink (Chapman and Fox, 1969; Tobach *et al.*, 1965).

Unsuccessful attempts have been made to learn how the sea hare uses ink in its natural environment. Carew and Kupferman (1974) and Kupferman and Carew (1974) reported on lengthy observations of *Aplysia californica* in a variety of habitats; inking was never observed. Furthermore, they did not observe an *Aplysia* being attacked by a predator. Inking occurred routinely, however, if the mollusks were roughly handled by the investigators. These results suggest, but do not prove, that the ink is a defense mechanism used only in rather extreme cases. The function of the ink would then be produced by its chemical composition rather than its optical properties.

Inking has been studied by the techniques of neurophysiology (*e.g.*, Byrne, 1981), and it was determined that an electric-shock stimulus must cross a high threshold before inking occurs (Carew and Kandel, 1977).

Chapman and Fox (1969) studied the correspondence between diet and the presence of ink in *Aplysia*. After inducing complete discharge of the ink by tactile stimulation, they fed the spent organisms either brown or red algae and found that only after feeding with red algae was the ink replenished. Rüdiger (1967) had shown that the major pigment in the ink has the structure of a monomethyl ester of phycoerythrobilin, a chromophore of the

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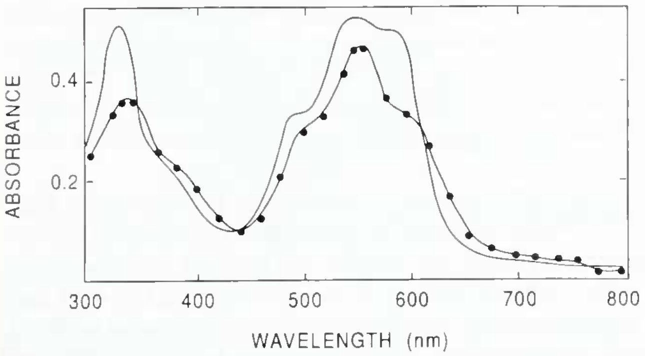

Figure 1. Absorption spectra of *Aplysia* inks. The visible spectra of inks from different animals may vary.

biliprotein phycoerythrin from red algae. Because brown algal seaweed are devoid of biliproteins, it is probable that the ink pigments are derived from the biliproteins of the red algae. Because these chromophores are covalently attached to protein via one or two thioether linkages to cysteine (for references, see MacColl and Guard-Friar, 1987), *Aplysia* must therefore cleave the chromophores off the biliproteins and store the pigments in the ink gland. Troxler *et al.* (1981) obtained some ink pigments that were still attached to a cysteine residue. The main pigment component was 90% free and 10% cysteine-bound. We have now investigated the identities of the other chromophores in the ink.

Materials and Methods

Aplysia californica was obtained from Marine Specimens Unlimited (Pacific Palisades, California) and were inked immediately upon arrival either by dissection or by physical stimulation. Care was taken to obtain ink that was free of secretion from the opaline gland. The thick, white, mucus-like substance from the opaline gland was easily observed if secreted, but usually in our protocols the ink gland secreted with no secretion of the opaline. Any ink contaminated by this substance was discarded. The ink was stored frozen until needed for our experiments.

The chromophore components of the ink were separated by two methods, thin layer chromatography and gel filtration chromatography. Thin-layer chromatography of the ink was performed on 5 × 10 cm Silica gel 60 F254 precoated plates. The chromatograms were developed with a solution of 50% benzene: 35% methanol: 15% ethyl acetate. The gel filtration experiments were carried out using Ultrogel AcA54 (LKB) with pH 6.0, 0.1 ionic strength, sodium phosphate buffer with 0.5 *M* NaCl added. Some plates were likewise developed in a second direction.

B-Phycoerythrin was isolated from the red alga, *Porphyridium cruentum*. The alga was grown in our laboratory, harvested, and stored frozen. Cells were broken in a French pressure cell, and the water-soluble proteins extracted into pH 6.0, 0.1 ionic strength, sodium phosphate buffer. The B-phycoerythrin was purified by ammonium sulfate fractionation (50% saturated ammonium sulfate), gel-filtration column chromatography on Sepharose 6B (Pharmacia), and chromatography on a hydroxylapatite column (Bio-gel HT, Bio-Rad). The ratios of the absorbance of B-phycoerythrin at its visible maximum compared with both the visible absorbance maxima of the other biliproteins and 280 nm were used to determine

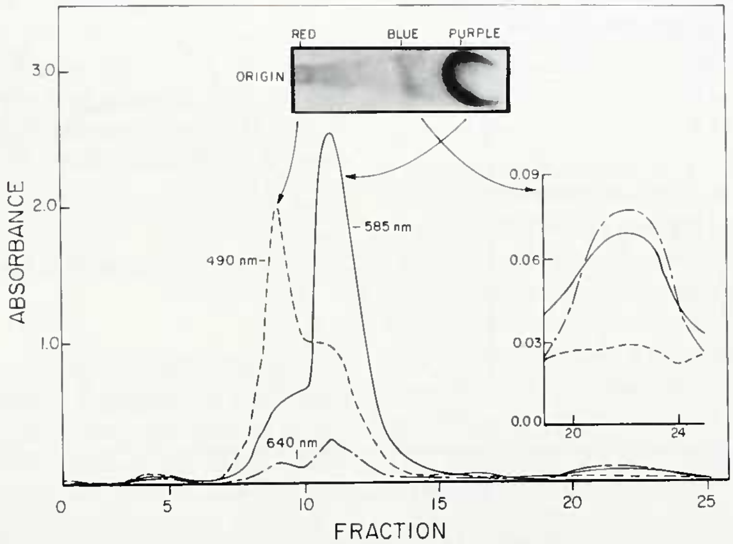

Figure 2. Chromatographic separation of *Aplysia* ink into three pigments. The TLC is shown as a photograph, and the Ultrogel elution pattern is plotted as absorbance *versus* fraction number. The inset shows the absorbances of these fractions on an expanded scale. Pools of ink from several sea hares were used in these experiments.

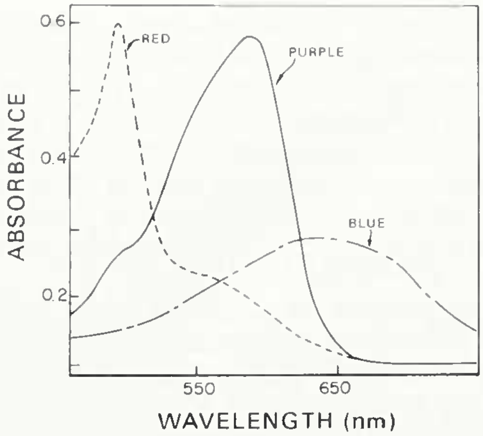

Figure 3. Visible absorption spectra of the three ink pigments. The solvent is sodium phosphate buffer, pH 6.0, with 0.50 *M* sodium chloride.

purity. The hydroxylapatite chromatography was performed as described previously (MacColl *et al.*, 1981), and the B-phycoerythrin eluted prior to the other biliproteins. Purified protein was dialyzed into distilled water, lyophilized, and stored in a refrigerator.

B-Phycoerythrin is composed of three subunits, α and β (17,500 molecular weight) and γ (30,000 molecular weight). The subunits were dissociated in 8.0 *M* urea, pH 3.0 and separated on a Sephacryl S-200 (Pharmacia) column in the same solvent. Fractions containing γ subunit were identified spectroscopically on the basis of its characteristic phycourobilin absorbance at 490 nm. The γ subunit has both phycourobilin and phycoerythrobilin chromophores while the α and β subunit have only phycoerythrobilins (see MacColl and Guard-Friar, 1987, for references). The fractions containing γ subunit were then rechromatographed on the same column to complete their purification.

C-Phycocyanin was isolated from the blue-green alga, *Phormidium luridum*, after treating the cells with the enzyme lysozyme. The protein was purified by ammonium sulfate precipitation, first with 50% followed by 35% saturated ammonium sulfate. Purified protein was dialyzed into distilled water, lyophilized, and stored in a refrigerator. C-Phycocyanin, which has only phycocyanobilin for chromophores, was refluxed in methanol overnight. The reflux mixture was filtered and the blue solution, containing phycocyanobilin, was evaporated to dryness. The absorption spectrum of the protein-free phycocyanobilin was obtained in acidic methanol. Absorption spectra were obtained at room temperature using a model 320 spectrophotometer (Perkin-Elmer).

Sodium dodecyl sulfate gel electrophoresis was performed using precast, 10–20% polyacryamide gradient gels (Geltech, Salem, Ohio). The ink was dialyzed into distilled water and lyophilized. The lyophilized material was suspended in pH 6.0, 0.1 ionic strength, sodium phosphate buffer with 1% sodium dodecyl sulfate. The reconstituted ink was treated with a sample buffer containing: 50 parts distilled water; 12.5 parts 0.3 *M* Tris HCl, pH 6.8; 10 parts glycerol; 20 parts (10% w/v) sodium dodecyl sulfate; 5 parts β-mercaptoethanol; and 2.5 parts (0.05% w/v)

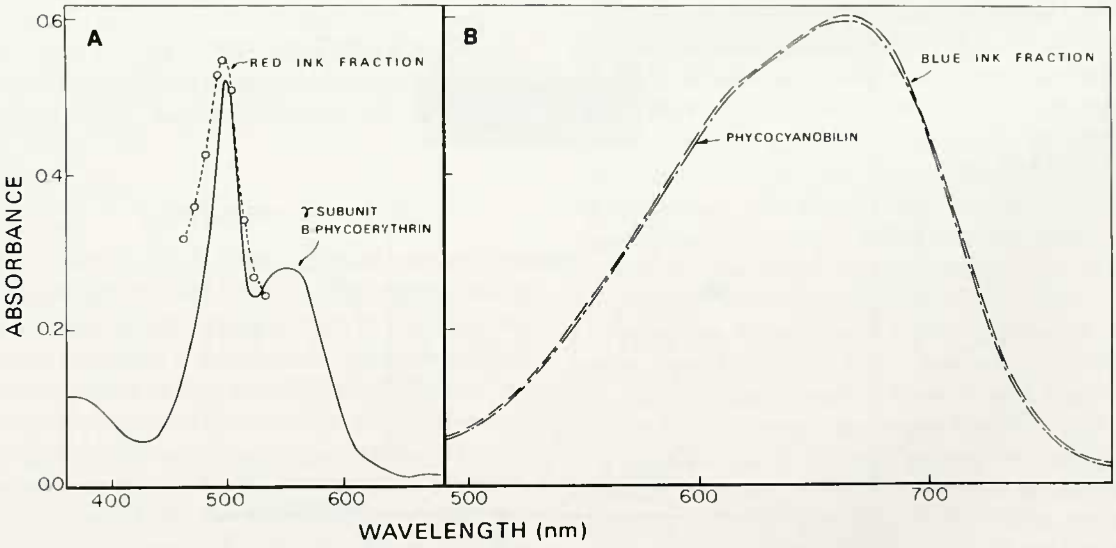

Figure 4. Spectroscopic comparisons of *Aplysia* pigments with biliprotein chromophores. (A) Comparison of red pigment with γ subunit of B-phycoerythrin. Phycourobilin has its absorption maximum near 490 nm, and phycoerythrobilin is near 560 nm. The solvent is 8.0 *M* urea, pH 3.0. This solvent minimizes the effect of the apoprotein on the spectrum of the bilin. (B) Comparison of blue pigment with phycocyanobilin cleaved from C-phycocyanin both in acidic methanol.

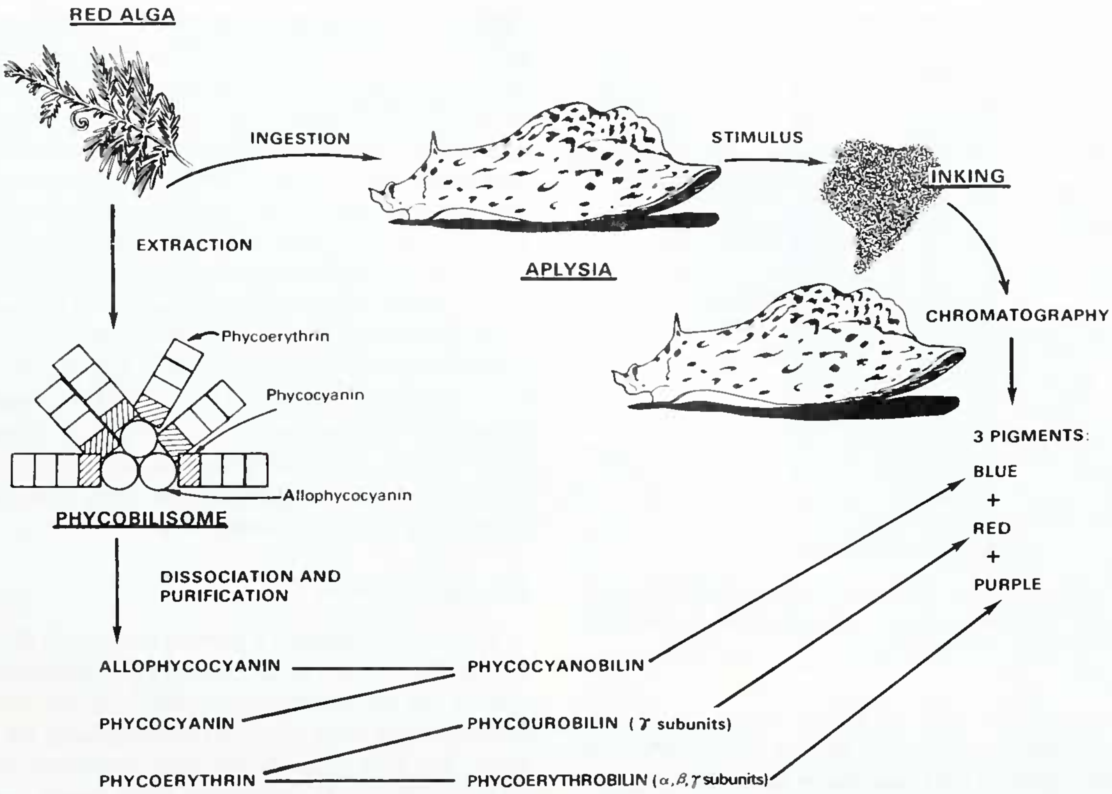
RED ALGA
INGESTION
STIMULUS
INKING
EXTRACTION
APLYSIA
CHROMATOGRAPHY
Phycoerythrin
Phycocyanin
Allophycocyanin
PHYCOBILISOME
3 PIGMENTS:
BLUE
+
RED
+
PURPLE
DISSOCIATION AND PURIFICATION
ALLOPHYCOCYANIN
PHYCOCYANIN
PHYCOERYTHRIN
PHYCOCYANOBILIN
PHYCOUROBILIN (γ subunits)
PHYCOERYTHROBILIN (α,β,γ subunits)

Figure 5. Schematic representation of derivation of pigments of *Aplysia* ink from phycobilisomes of a hypothetical red alga. Although not indicated on the diagram, B-phycoerythrin has phycourobilin only on the γ subunit, while R-phycoerythrin has phycourobilin on both the γ and β subunits. C-Phycocyanin has only phycocyanobilins, while R-phycocyanin has both phycocyanobilin and phycoerythrobilin. (For references, see MacColl and Guard-Friar, 1987.)

Bromophenol Blue. The final concentration of ink was 100 μg in the 20 μl of heated sample which was applied to each lane. The gels were stained with Coomassie blue, and destained with 50% distilled water; 40% methanol; 10% acetic acid. The gels were then stored in a 5% glycerol solution. Standards used for molecular weight calibration were: bovine serum albumin, ovalbumin, chymotrypsinogen, and lysozyme.

Results and Discussion

Chromophore content

Visible absorption spectra of *Aplysia* inks (Fig. 1) show a number of bands. The relative intensities of the various band maxima vary with ink source; two typical results are shown (Fig. 1). Earlier studies on the chromophore content of the ink have yielded variable results (Christomanos, 1955; Winkler, 1959; Chapman *et al.*, 1967; Lederer and Huttrer, 1942; Schreiber, 1929; Nishibori, 1960). We have, therefore, chosen to separate the ink chromophores by two entirely distinct methods: thin-layer (non-aqueous) and gel filtration (aqueous, pH 6.0) chromatography. Thin-layer chromatography (TLC) separated the ink into three colored components. The fastest-migrating ($R_f = 0.53$) was purple; the middle component ($R_f = 0.40$) was blue; and the third, which remained near the origin ($R_f = 0.07$), was red. When a TLC plate was run in two dimensions, a yellow component was also observed in the vicinity of the blue. Gel filtration produced very similar results; the brownish-red material eluted first, followed by purple, and finally by a light blue band (Fig. 2). The major purple and red fractions were rechromatographed on the same Ultrogel column, and the absorption spectra of the three purified components were obtained (Fig. 3). The red pigment showed a prominent absorption band at 490 nm, the purple maximum was at 585 nm, and the blue at 640 nm.

The absorption spectrum of the brownish-red component, which was found by both thin-layer and Ultrogel chromatography, was compared with the absorption spectrum of a biliprotein chromophore—phycourobilin—obtained from β-phycoerythrin (Fig. 4A). Phycourobilin is covalently attached to the γ subunits of B-phycoerythrin, which was purified by chromatography on Sephacryl

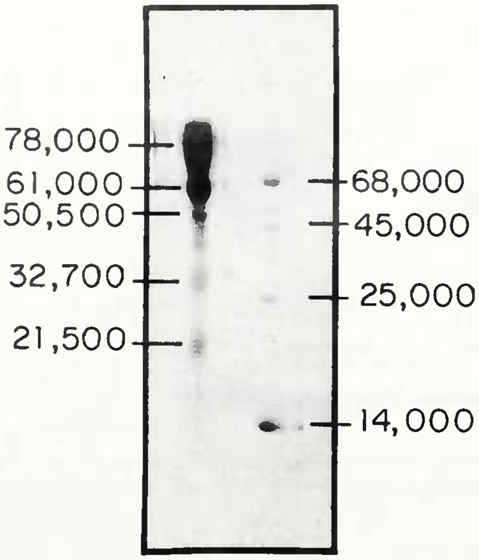

Figure 6. Sodium dodecyl sulfate gel electrophoresis results of *Aplysia* ink. On the right are molecular weight standards, and on the left are the polypeptides from a pool of ink.

S-200 in the presence of 8.0 *M* urea at pH 3.0. Phycourobilin has its absorption maximum near 490 nm, and its absorption spectrum shows also that phycoerythrobilin also occurs on this subunit. Considering these two differences—that phycourobilin is covalently attached to a polypeptide and that it occurs together with phycoerythrobilin on that subunit—the agreement between the absorption spectra of phycourobilin and the red ink component is satisfactory. The acidic urea solvent minimizes the effects of apoprotein on the spectra of the bilins.

The blue component of the ink was also purified by column chromatography. Its spectrum is identical to that of authentic phycocyanobilin (Fig. 4B), which was obtained by methanol refluxing of C-phycocyanin. The structure of phycocyanobilin has been determined by Cole *et al.* (1967). The spectrum of phycocyanobilin is highly dependent on solvent, and near neutral pH the absorption maximum is blue shifted to around 600 nm. The pH of the ink is about 6.54 ± 0.35.

The three pigments of *Aplysia* ink therefore appear to be derived, apparently without extensive chemical modification, from three algal biliprotein chromophores. The precise diet of the *Aplysia* used in our studies is unknown, but we can assume that a representative red alga is a component of the diet. In this seaweed a typical selection of biliproteins—B-phycoerythrin (or R-phycoerythrin), C-phycocyanin (or R-phycocyanin), and allophycocyanin—would be arranged in phycobilisomes (Fig. 5). The C-phycocyanin and allophycocyanin have as their chromophore phycocyanobilin, which becomes the blue ink component. B-Phycoerythrins (or R-phycoerythrins) have both phycoerythrobilin and phycourobilin, which become the purple and red ink components, respectively. Several phycoerythrobilins per phycourobilin can be found on each phycoerythrin aggregate (for B-phycoerythrin the ratio is 3.5 to 1). Most red algal phycobilisomes have more phycoerythrin than the sum of phycocyanin plus allophycocyanin (Fig. 5), and each phycoerythrin aggregate has more chromophores than do similarly sized aggregates of allophycocyanin or C-phycocyanin. The predominance of the purple component over the blue is thus a function of the structure of the red algal phycobilisome. In addition, Chapman and Fox (1969) found some phycocyanobilin to be selectively localized in the skin of the *Aplysia*. Variability in the color of the ink (*e.g.*, Winkler, 1959) is most likely related to the feeding of individuals on different types of red algae since the various algae show biliprotein diversity in their phycobilisomes.

Polypeptide content

Studies examining the protein content of the ink were conducted. Sodium dodecyl sulfate gel electrophoresis was carried out on proteins extracted from the ink (Fig. 6). Several bands were observed after staining for polypeptides. The two main bands have molecular weights of 78,000 and 61,000. The other three minor bands have molecular weight values of 50,500, 32,700, and 21,500. Biliproteins have polypeptides ranging from 15,000 to 22,000 (MacColl and Guard-Friar, 1987), and they are apparently not major ink constituents. Additional immunochemical experiments are needed to discover whether any biliproteins are present in the ink.

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The Anatomy and Morphology of the Adult Bacterial Light Organ of *Euprymna scolopes* Berry (Cephalopoda:Sepiolidae)

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Abstract. The sepiolid squid, *Euprymna scolopes*, has a bilobed luminous organ in the center of the mantle cavity, associated with the ink sac. Luminous bacterial symbionts (*Vibrio fischeri*) are housed in narrow channels of host epithelial tissue. The channels of each lobe of the light organ empty into a ciliated duct, which is contiguous with the mantle cavity of the squid. Surrounding the symbiotic bacteria and their supportive host cells are host tissues recruited into the light organ system, including a muscle-derived lens and thick reflector that appear to permit the squid to control the quality of bacterial light emission.

Introduction

Euprymna scolopes is a small (average adult mantle length, approximately 25 mm) benthic squid indigenous to Hawaii (Berry, 1912; Singley, 1983). As a nocturnal predator, it buries in the sand during the day and forages at night over shallow-water sand flats. *E. scolopes* shares with a number of other sepiolids the characteristic of culturing marine luminous bacteria in a symbiotic, light organ association (Kishitani, 1932; Boletzky, 1970; Herring *et al.*, 1981). The structural and functional relationships of the various tissues making up the complex light organ, as well as several behavioral observations (Moynihan, 1983; McFall-Ngai, pers. obs.), suggest that the luminescence is used by the squid in counterillumination (*i.e.*, production of ventrally directed luminescence to camouflage its silhouette against background light) and in startle displays (Herring *et al.*, 1981; Moynihan, 1983; McFall-Ngai, pers. obs.).

The present paper describes the anatomical and ultrastructural relationships of *E. scolopes* and its bacterial partner, *Vibrio fischeri*, in the adult light organ association. In providing a baseline understanding of the tissue relationships in the mature system, this report complements emerging studies on the initiation, development, and specificity of mutualistic associations between higher animals and bacteria (McFall-Ngai and Ruby, 1989; Ruby and McFall-Ngai, 1989).

Materials and Methods

Specimens of *Euprymna scolopes* were collected with dipnets from shallow (<1 m) water in Kaneohe Bay on the island of Oahu, Hawaii, shortly after dusk during March 1988, 1989, and December 1989. The animals were transported to recirculating aquaria at the University of Hawaii, Manoa campus, or to running-seawater tables at the Hawaiian Institute of Marine Biology (University of Hawaii) on Coconut Island in Kaneohe Bay, where they were maintained until used in experiments.

Animals were fixed for light microscopy in 10% formaldehyde buffered with 0.5 *M* sodium phosphate, pH 7.4. They were then dehydrated through a graded ethanol series and infiltrated with propylene oxide before transfer to unaccelerated Spurr (Spurr, 1969). The samples remained in unaccelerated Spurr for one week at room temperature, followed by 24 h in accelerated Spurr at room temperature. Samples were then embedded in fresh accelerated Spurr in an oven at 67°C for 48 h.

The general construction of the light organ, and information from the literature on fixation procedures for transmission electron microscopy of similar tissues (Herring *et al.*, 1981), indicated that penetration of the light organ by fixatives might be a problem. Therefore, we conducted a series of experiments to determine the optimal fixation conditions. We fixed both whole and subdivided

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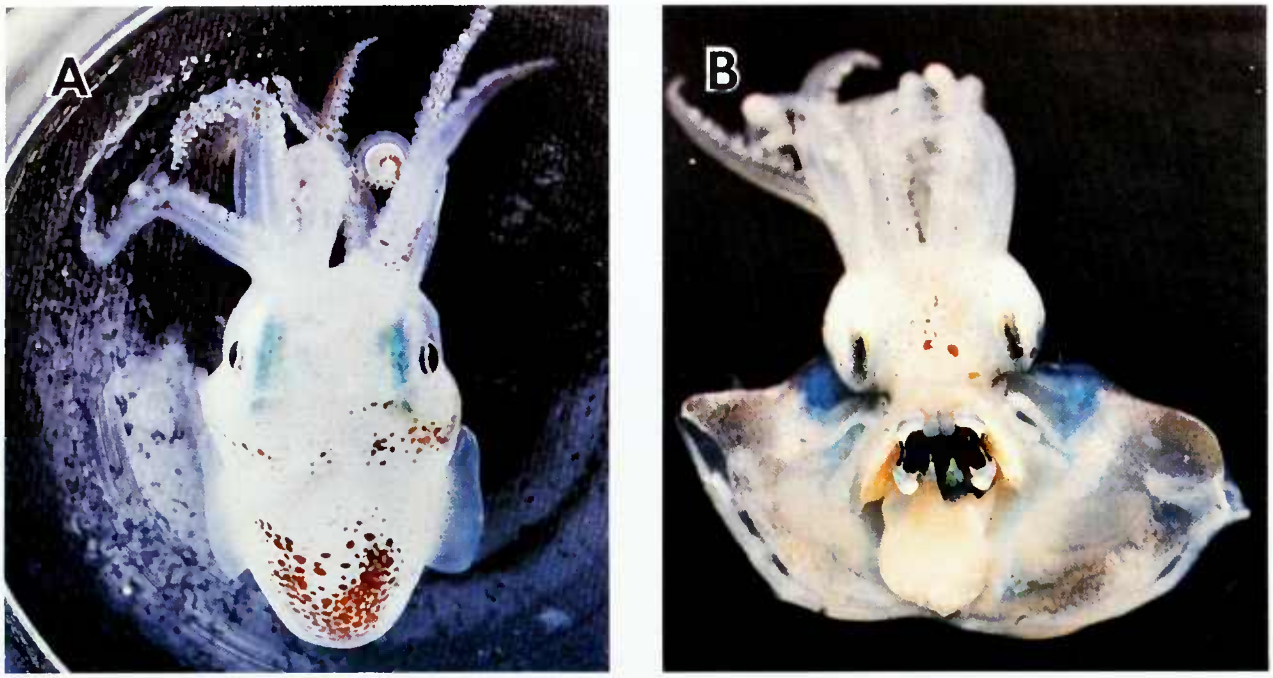


Figure 1. *Euprymna scolopes.* A. Dorsal view of adult specimen (mantle length approximately 20 mm). B. Ventral dissection of same specimen exposing the light organ. The light organ appears as the black and white kidney-shaped lobes overlaying the yellow digestive gland.

light organs from small and large specimens for either 60, 75, or 90 min, or for 6, 12, or 18 h. In addition, we tested the quality of ultrastructural detail obtained with the following fixatives: 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer with 0.40 *M*, 0.45 *M*, or 0.50 *M* NaCl, pH 7.4; and 2.5% glutaraldehyde in 0.2 *M* sodium phosphate buffer with 0.14 *M* NaCl, pH 7.4. All samples were postfixed in 1% osmium tetroxide in the fixative buffer and were processed similarly to those samples fixed for histology (see above).

Our experiments with tissue preparation for electron microscopy showed that the best preservation resulted from long fixation times (>6 h) in 0.1 *M* sodium cacodylate buffer with 0.45 or 0.50 *M* NaCl. Some components of the light organ showed enhanced fixation with 0.45 *M* NaCl while others showed similar improvements with 0.50 *M* NaCl (see figure legends).

Results

The fully developed light organ of *Euprymna scolopes* is a bilobed structure that occupies a significant portion of the mantle cavity (Fig. 1A, B). Ten specimens, ranging in size from 4.0 mm to 25 mm in dorsal mantle length (ML), were used for microscopy. The light organ ranged in anterior-posterior length from 1.5 mm in the smallest animal to 7.5 mm in the largest individual. Although ongoing studies in our laboratory have shown that the development of the *E. scolopes* light organ involves a set of complex stages, all components of the light organ are present and appear mature in juveniles as small as 4 mm ML.

Histological analysis of the light organ of this species (Fig. 2) revealed that the tissues and their anatomical relationships were similar to those described in the light organ system of another sepiolid, *Sepiola atlantica* (Her-

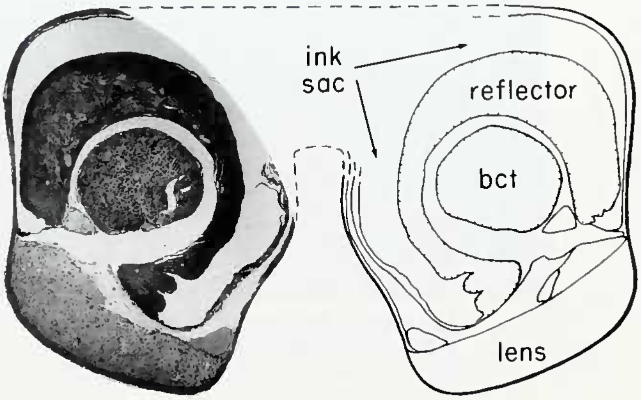


Figure 2. Light micrograph and opposing diagram of a typical 1-μm histological cross-section through the light organ of *Euprymna scolopes* showing the various associated tissues. The ink is lost from the ink sac during dissection, and shrinkage that occurs during fixation and embedding procedures causes the tissue containing the bacteria to pull away from the reflector. 20×. (bct, tissue containing bacteria).

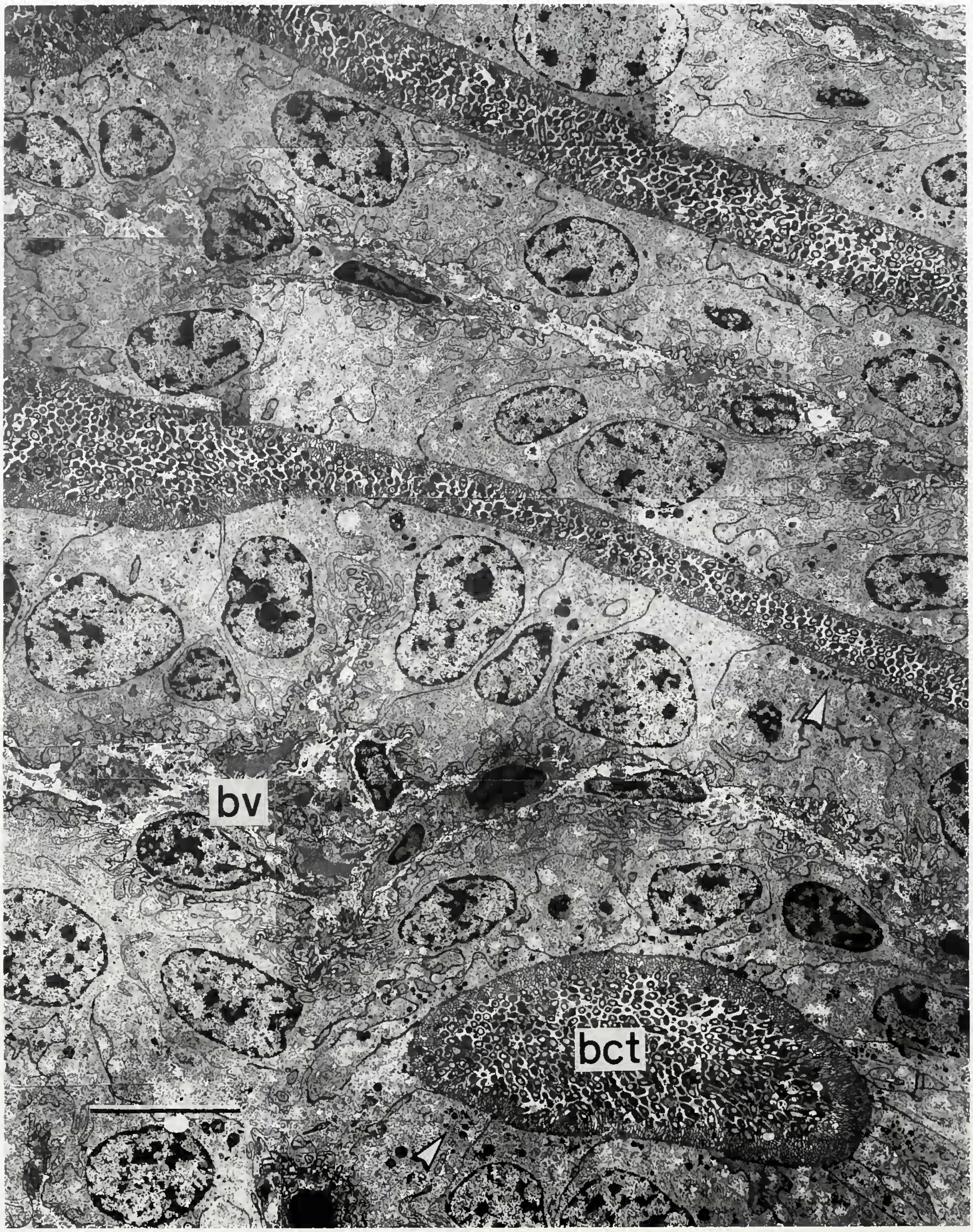


Figure 3. Composite transmission electron micrograph of the bacteria-containing central core of the light organ of *Euprymna scolopes*. Numerous electron-dense vesicles (arrows) are concentrated in portions of the host cell adjacent to the tubules that contain bacteria. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.45 *M* NaCl. Bar scale = 10 μm. (bct, bacteria; bv, blood vessel)

ring *et al.*, 1981). The bacteria occur in animal tissue that is surrounded by a thick reflector, which is, in turn, surrounded by diverticula of the ink sac. In some preserved specimens, the medioventral portion of the reflector was pulled back, and ink in the ventral portions of the ink sac shunted medially (see Fig. 2). Histological analysis of a large number of specimens showed considerable variability in the positions of the reflector and ink sac in re-

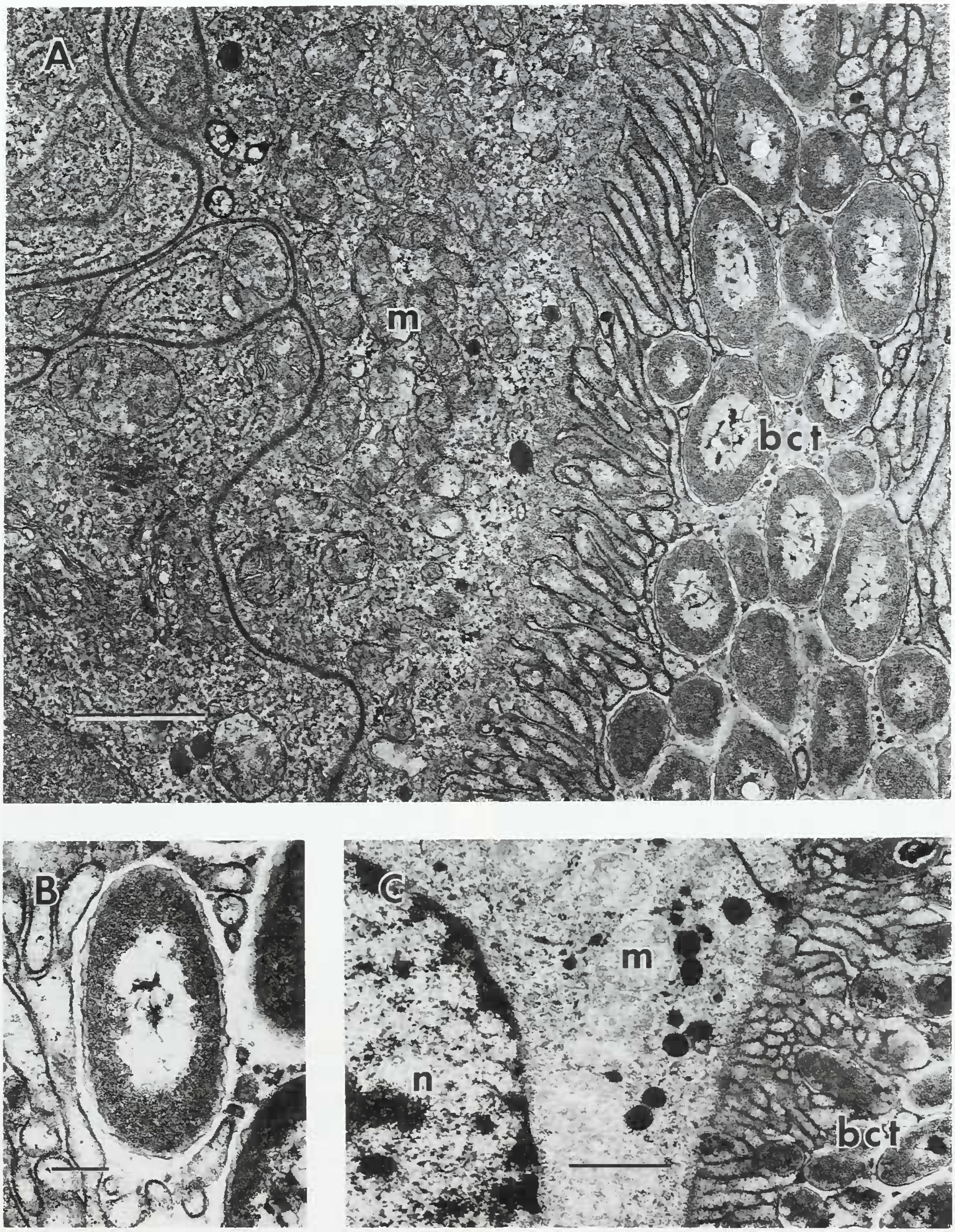


Figure 4. High magnification transmission electron micrographs of the light organ tissue that contains the bacteria. A. Abundant mitochondria occur in the host cell adjacent to the microvillous border lining the tubule that houses the bacteria. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.50 *M* NaCl. Bar scale = 1 μm. B. *Vibrio fischeri* cell as it appears in symbiosis with squid tissue. This single bacterium, although extracellular, appears almost completely surrounded by host cell membrane. Primary fixative is the same as above. Bar scale = 0.25 μm. C. The lower osmolarity of this buffer resulted in poor fixation of the mitochondria, but better fixation of the electron-dense vesicles, both of which appear in abundance in the portion of the animal cell adjacent to the microvillous border that lines the tubules containing bacteria. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.45 *M* NaCl. Bar scale = 1 μm. (bct, bacteria; m, mitochondria; n, nucleus)

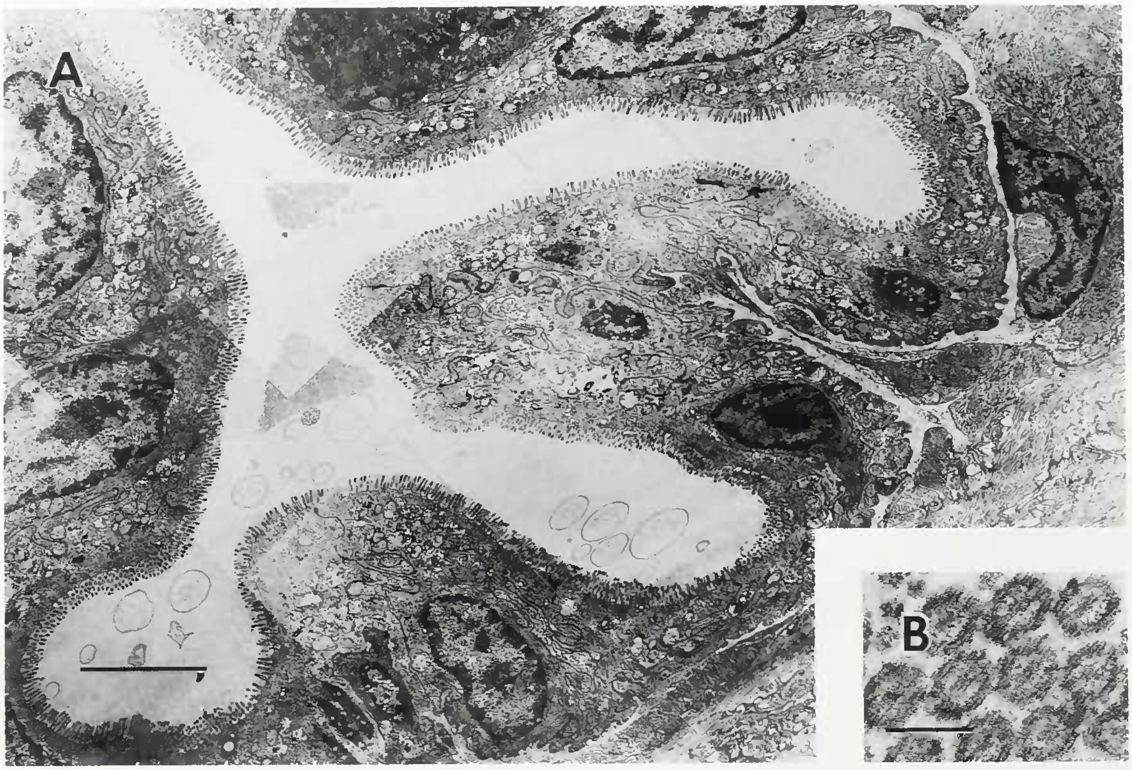
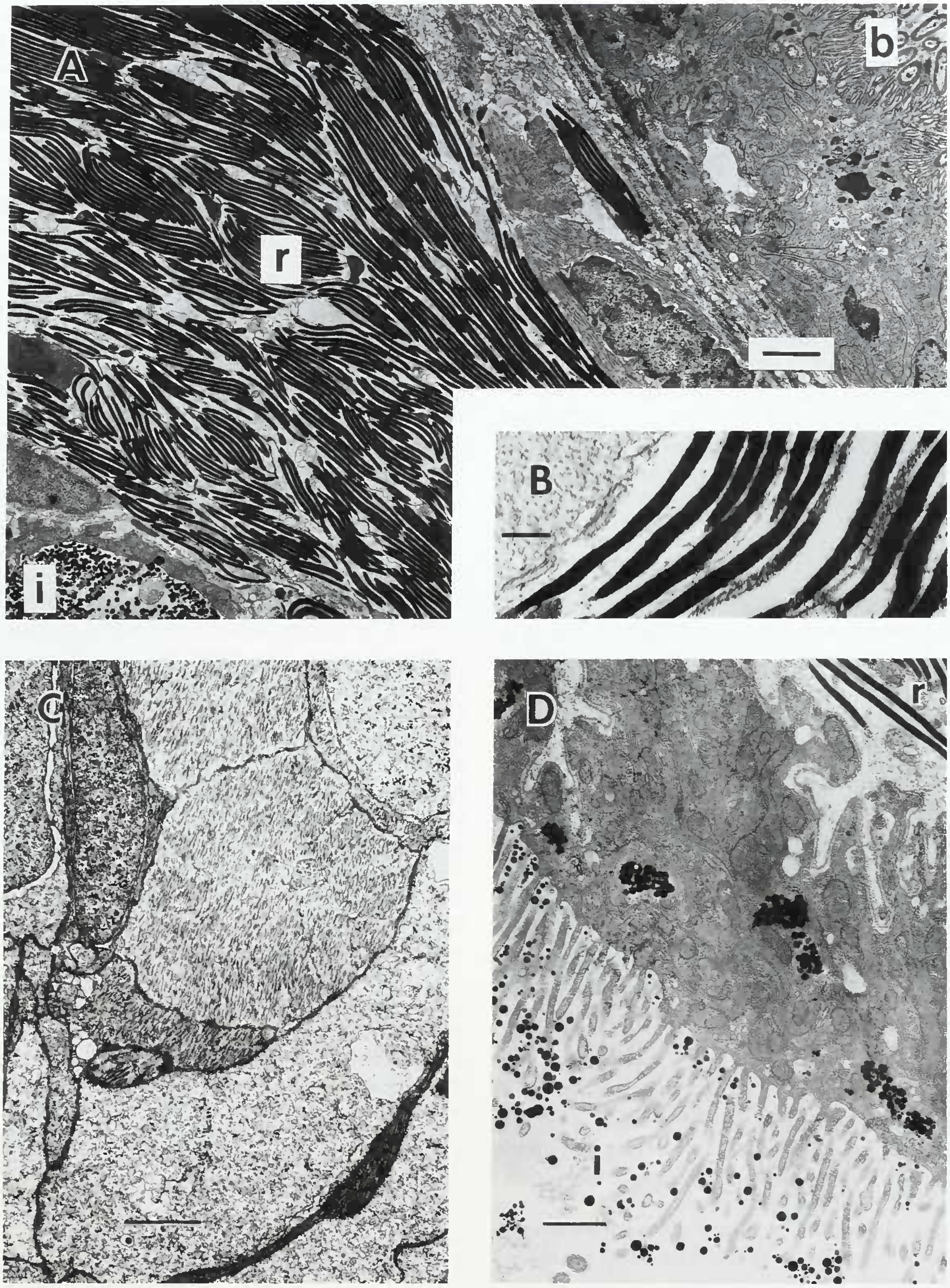


Figure 5. Composite electron micrograph of the ciliated duct of the light organ of *Euprymna scolopes.* A. The composite resulted from a transverse section of the light organ and shows the ciliated duct that leads to the lateral pore on each lobe. Branches of the duct shown in this micrograph are contiguous with the tubules that contain bacteria. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.45 *M* NaCl. Bar scale = 5 μm. B. High magnification showing the cilia in cross-section. Primary fixative as above. Bar scale = 0.25 μm.

lation to the tissue that contains the bacterial symbionts. These data, coupled with observations of the behavior of light organ tissues of anesthetized, dissected animals, indicate that the expression of light is controlled by movements of both the reflector and ink sac. The entire ventral surface of the light organ is covered by a thick, transparent lens, the outer edge of which is continuous with the ink sac lining (Fig. 2). Microscopic examination of the intact light organ revealed a pore on the lateral face of each light organ lobe. Histological sections through this area revealed that the pore is continuous with the light organ tissue that contains bacteria (data not shown).

Low magnification transmission electron micrographs of light organ tissue that contains bacteria (Fig. 3) reveal that the bacteria occur in narrow channels, usually only a few bacteria in width, in most portions of the light organ. The narrow channels are surrounded by a single layer of animal cells, which are surrounded by a layer of blood vessels and other connective tissue elements. This pattern is repeated through the bacterial core tissue. Microvilli from the epithelial cells of the animal invest the bacterial culture. Electron-dense vesicles occur in the portions of the animal cells adjacent to the bacterial culture, suggesting the exchange of materials. Observations at higher magnifications of the animal cell/bacterium interface (Fig. 4) revealed that, in addition to electron-dense vesicles,

Figure 6. Accessory structures of the light organ of *Euprymna scolopes.* A. Transmission electron micrograph of the light organ with some of the structures associated with light modulation. This section is through a particularly narrow portion of the reflector (r) so that several layers of the system are viewed. The reflector is closely associated with the lining of the ink sac (see also 6D) and surrounds much of the central core tissue, which contains the bacterial symbionts. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.45 *M* NaCl. Bar scale = 2 μm. B. High magnification micrograph of the reflector showing the membrane bound platelets. Primary fixative same as above. Bar scale = 0.25 μm. C. Transmission electron micrograph of the lens exposing its lack of detailed cell structure but indicating its



development from muscle-derived tissue. Note the numerous, thin, aligned filaments filling most of the cells. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.5 *M* NaCl. Bar scale = 2 μm. D. Transmission electron micrograph of the lining of the ink sac showing its ciliated ink-producing cells and its close association with the reflector. Primary fixative used was same as for 6A. Bar scale = 1 μm. (b, bacteria; i, ink sac; r, reflector) high densities of mitochondria exist in portions of the animal cell adjacent to the bacteria. Tubules containing bacteria empty into a common, ciliated duct that is connected with the pore on the lateral face of each light organ lobe (Fig. 5). This duct provides a direct connection between the bacterial culture and the mantle cavity of the animal.

The tissue containing bacteria is surrounded by reflective tissue, which is itself enclosed by the ink sac (Fig. 6A). The reflector is made up of cells containing platelets oriented perpendicularly to the bacterial culture, and has a similar structure to that reported for the reflective tissues of other cephalopods (Arnold *et al.*, 1974; Brocco and Cloney, 1980; Cloney and Brocco, 1983). The width of the reflector is several dozen to hundreds of platelets thick, depending on the location within the light organ. These electron-dense reflector platelets, which appear to be membrane bound (Fig. 6B), averaged 100 nm wide with cytoplasmic spacing that varies from 50 nm to 200 nm. Because of shrinkage during preparation, however, this may not represent the actual spacing. The ventral portion of the reflector abuts the lens of the light organ (see Fig. 2), the ultrastructural characteristics of which suggested that it was derived from muscle tissue (Fig. 6C). The cells of the lens had little structural detail except for the presence of numerous, thin, aligned elements. The ink sac lining appeared as a mitochondrion-rich tissue with electron-dense vesicles, presumably packaged ink (Fig. 6D).

Discussion

Progress toward an understanding of developmental processes in higher animal/bacterial mutualisms has been slow because of the lack of tractable experimental systems. Unlike plant mutalisms, such as the leguminous plant/*Rhizobium* symbiosis (Long, 1989), animal mutualisms usually involve a variety of different species of microorganisms in a single host, or are characterized by a host that cannot live axenically or by symbionts that cannot be cultured. One of the few higher animal/bacterial mutualisms the study of which does not suffer from these drawbacks, is the symbiotic relationship between the sepiolid squid, *Euprymna scolopes*, and its luminous bacterial symbiont, *Vibrio fischeri*. The squid host, which can be raised in the laboratory (Arnold *et al.*, 1972; pers. obs.), hatches without its luminous symbiont (Wei and Young, 1989). *V. fischeri*, which occurs freeliving in the water and is readily culturable (Ruby and McFall-Ngai, 1989; Boettcher and Ruby, 1990), is picked up by the newly hatched squid within hours after hatching (Wei and Young, 1989).

A prerequisite to the studies of the development of the light organ is a description of the morphology and anatomy of the adult association. Although the adult light organ of *E. scolopes*, described here, is similar to that of other sepiolids (Kishitani, 1932; Herring *et al.*, 1981), some ultrastructural differences have emerged. Herring *et al.* (1981) reported that the bacteria of another sepiolid squid, *Sepiola atlantica*, are loosely associated with the animal cells, which lack microvilli. In contrast, the bacterial culture in the light organ of *E. scolopes* is in intimate contact with the microvillous border of the host cells. Caution must be exercised in interpreting these differences, which could be due to differences in the quality of fixation.

The high concentrations of mitochondria in the portions of the animal cells adjacent to the bacteria may be of particular significance in the physiology and metabolic dynamics of the light organ association. Monocentrid fishes also have light organs containing an abundance of mitochondria in the cells next to the bacterial symbionts (Tebo *et al.*, 1979). Under laboratory culture conditions of low oxygen, *Vibrio fischeri* grows poorly, but luminesces brightly and excretes pyruvate (Ruby and Nealson, 1976; Nealson and Hastings, 1977). The ultrastructure of the monocentrid light organ and the physiology of the bacteria in culture has led to a model for this symbiosis (Nealson, 1979). The model holds that pyruvate excreted by the bacteria fuels the mitochondria, the respiratory activity of which keeps the oxygen tension low around the bacterial culture, thus promoting the slow growth but high luminescence of the bacteria in the association. However, while the light organ tubule cells of *E. scolopes* also have high densities of mitochondria, the symbiotic strain of *V. fischeri* they surround does not show enhanced luminescence under low oxygen tensions (Boettcher and Ruby, 1990). Thus, physiological behavior of the *E. scolopes* bacteria in culture and the ultrastructural characteristics of the light organ are inconsistent with the model developed for the monocentrid fish symbiotic association.

During the development and ontogeny of a complex light organ, such as that of *Euprymna scolopes*, tissues must be recruited and modified to form the various components of the organ. Not only must tissue be adapted so that the squid can efficiently use the light produced by the bacteria, but the light organ and the bacterial culture must be supported by recruited vascular and nervous tissue. Further, the squid host must produce a site that promotes the growth and luminescence of the native symbiotic bacterium, while excluding other bacterial species. How these processes are orchestrated to create the complex adult structure, and the part played by the bacteria in the morphogenesis of the light organ, should be revealed through experimental manipulations of the developing system.

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Patterns of Stimulated Bioluminescence in Two Pyrosomes (Tunicata: Pyrosomatidae)

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Abstract. Pyrosomes are colonial tunicates that, in contrast with typical luminescent plankton, generate brilliant, sustained bioluminescence. They are unusual in numbering among the few marine organisms reported to luminesce in response to light. Each zooid within a colony detects light and emits bioluminescence in response. To investigate the luminescence responsivity of *Pyrosoma atlanticum* and *Pyrosomella verticillata*, photic, electrical, and mechanical stimuli were used. Photic stimulation of 1.5×10^9 photons $\cdot$ s^{-1} $\cdot$ cm^{-2}, at wavelengths between 350 and 600 nm, induced bioluminescence, with the maximum response induced at 475 nm. The photic-excitation half-response constant was 1.1×10^7 photons $\cdot$ s^{-1} $\cdot$ cm^{-2} at 475 nm for *P. atlanticum; P. verticillata* had a significantly higher half-response constant of 9.3×10^7 photons $\cdot$ s^{-1} $\cdot$ cm^{-2}. Individual zooids within a colony, however, appeared to have different half-response constants. Stimulus strength influenced recruitment of zooids and, in turn, luminescent duration and quantum emission. Image intensification revealed saltatory propagation of luminescence across the colony, owing to photic triggering among zooids. Repetitive, regular mechanical or electrical stimulation elicited rhythmic flashing characterized by alternating periods of high and low light intensities.

Introduction

Pyrosomes are holoplanktonic colonial tunicates found at depths to 1000 m (Soest, 1981). Their remarkable capacity to luminesce and their occasional presence in very large numbers at the ocean surface occasioned T. H. Huxley to write in his diary in 1849: "I have just watched the moon set in all her glory, and looked at those lesser moons, the beautiful Pyrosoma, shining like white-hot cylinders in the water" (Huxley, 1936).

Colonies may be of highly variable size, reaching lengths of 30 m in some species (Griffin and Yaldwyn, 1970), owing to their growth habit of budding successive rings of zooids around the periphery of an elongating cylinder. The zooids are arranged so that their exhalent currents are conducted into the hollow core of the cylinder to generate a communal locomotor current. Each zooid contains a pair of luminescent organs, bilaterally flanking the incurrent siphon, and lying at the periphery of the colonial cylinder (Panceri, 1873). The organs are external to the pharyngeal epithelium, but protrude into the pharyngeal cavity (Mackie and Bone, 1978). Closely packed cells in the luminescent organs are filled with luminous organelles, which may be intracellular luminescent bacteria (Pierantoni, 1921; Neumann, 1934; Buchner, 1965; Mackie and Bone, 1978). Bacterial luciferase activity similar to that of the luminescent bacteria *Photobacterium* has been found in *Pyrosoma* sp. (Leisman *et al.*, 1980).

The mechanism of luminescence propagation within the colony is not neural. Neither innervation nor an epithelial conduction pathway is in evidence (Mackie and Bone, 1978). Photic stimuli are presumably received by a photoreceptor lying just above the brain, triggering brain-induced arrests of gill basket cilia (Buchner, 1965; Mackie and Bone, 1978). This ciliary arrest, and the concomitant gill collapse, might reduce blood flow and decrease the supply of oxygen or metabolites to the light organ, thereby indirectly controlling light emission (Mackie and Bone, 1978; Mackie, 1986).

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Pyrosomes have the remarkable ability to detect external light flashes and to respond by luminescing (Polimanti, 1911). Colonies respond to conspecifics (Burghause, 1914) and simulated bioluminescence (Mackie and Bone, 1978). Localized stimulation of one area of a colony produces a wave of light that travels across the colony from the point of stimulation (Panceri, 1873). Intact tissue connections are not necessary among zooids, indicating that wave propagation within the colony may occur photically (Burghause, 1914; Mackie and Bone, 1978).

Luminescence may also be photically induced in other organisms, including ctenophores (Lábas, 1980), ostracods (Tsuji *et al.*, 1970), copepods (Lapota *et al.*, 1986), euphausiids (Kay, 1965; Tett, 1969, 1972), and a decapod shrimp (Herring and Barnes, 1976). Pulsed, colored light at 700 m *in situ* enhanced bioluminescence activity from unidentified organisms (Neshyba, 1967). Some of these organisms undergo inhibition of their luminescence when exposed to constant, "bright" illumination (Burghause, 1914; Nicol, 1960).

The organisms used in this study were *Pyrosoma atlanticum* Peron, a cosmopolitan form, and *Pyrosomella verticillata* (Neumann), which occurs in tropical and subtropical waters (Soest, 1981). Although we investigated bioluminescence produced by photic, electrical, and mechanical stimulation, photic stimulation was our major concern because it is the least well understood among the bioluminescence excitatory modes, has never been investigated quantitatively, and has significance in the interpretation of the roles of bioluminescence in the behavior of marine animals. In our work, photic stimuli varying in wavelength and irradiance were related to their bioluminescent responses and compared with the effects of other stimulus modes. The pattern of the luminescent wave and the mode of its transmission through the colony are discussed. A preliminary report of this work has appeared (Bowlby and Case, 1988).

Materials and Methods

Specimen collection

Mature specimens of *Pyrosoma atlanticum* and *Pyrosomella verticillata* were studied during July 1986 and 1987, aboard the R.V. *New Horizon* off the southwest coast of Oahu, Hawaii. Collections were made at approximately 21° N 158° W, with an opening-closing Tucker Trawl (length, 30 m; mouth, 10 m^2). The trawl was equipped with an insulating cod end (Childress *et al.*, 1977), towed at depths ranging from 400 to 800 m, and brought to the surface every 4 to 6 hours. Specimens were sorted under ambient light, and maintained in darkness in 12°C seawater for 8 to 24 hours. The colonies studied ranged in length from 1.4 to 7.7 cm; surface area was calculated from measurements of colonial length and diameter. Colonies up to 30 cm in length were captured, but were not included in the investigation due to space limitations in the experimental apparatus.

Experimental procedure

Individual colonies were placed in covered Plexiglas chambers holding 25 to 125 ml of filtered seawater. The dimensions of the chamber substantially exceeded those of the colony to minimize luminescence induced by contact with the container. A 25-cm diameter integrating sphere, coated internally with white Polane polyethylene paint (97% reflectance at 500 nm), surrounded the animal to insure maximal reflectance and detection of bioluminescence irrespective of orientation (Latz *et al.*, 1987). Bioluminescence was detected by a photon counting photomultiplier tube (RCA Model 8850), which viewed the interior of the sphere through a 4.5-cm diameter port. A baffle between the source and detector only allowed light that had undergone multiple reflections within the sphere to be measured. This apparatus provides a directionally unbiased, quantifiable measure of bioluminescence from non-isotropic sources. Radiometric calibrations were made with an Optronics Laboratory Model 310 multifilter calibration source referenced to an NBS standard. At sea, the system calibration was maintained with a C^{14} phosphor referenced to the Optronics source. The calibration corrected for the spectral responsivity of the sphere and photomultiplier tube, as well as for the bioluminescence spectrum of both pyrosome species, as measured by an optical multichannel analyzer (Widder *et al.*, 1983).

The photomultiplier signal was monitored for 40 to 200 s with a Norland Model 5400 multichannel analyzer (MCA) and stored on a diskette in a microcomputer for subsequent analysis. Temporal resolution ranged from 10 to 50 ms per channel.

Bioluminescence was stimulated by light, electrical, and mechanical excitation. Electrical stimuli (0.5–50 Hz, 5 ms duration, 50 V) from a Grass S48 stimulator were delivered by tungsten electrodes projecting into the chamber. A 1-cm diameter fiberglass rod driven by a solenoid to produce a displacement of 1 cm in 0.5 s applied mechanical stimulation. In some trials the colony was stimulated with the rod manually until bioluminescence was no longer produced. Photic stimuli, produced by a Bausch and Lomb monochromator with a tungsten light source, were delivered through a 5-mm diameter fiber optic into the sphere. Stimuli entering the sphere were deflected by a stimulus baffle placed at 45° to the fiber optic, providing a uniform stimulus illumination over the entire colony. Stimulus wavelength (FWHM = 21 nm) was either varied between 350 nm and 800 nm (in 25 nm

Table I

Kinetics and intensities of pyrosome flashes stimulated at effective wavelengths (350–550 nm) and irradiances (6.8×10^6 to 4.3×10^{10} photons·s^{-1}·cm^{-2})

Species	Latency (s)	Rise time (s)	98% Duration (s)	Maximum flux (photons·s^{-1})	Mean emission (photons·s^{-1})	Quantum emission (photons·$flash^{-1}$)
Pyrosoma atlanticum (n = 6)	1.4 ± 0.2	4.9 ± 1.6	16.0 ± 3.8	1.2×10^{11} ± 1.0×10^{11}	4.8×10^{10} ± 3.8×10^{10}	1.4×10^{12} ± 1.1×10^{12}
Pyrosomella verticillata (n = 9)	1.4 ± 0.1	4.3 ± 1.1	11.6 ± 2.9	1.1×10^{10} ± 4.3×10^9	4.5×10^9 ± 2.1×10^9	1.0×10^{11} ± 5.0×10^{10}

Values represent the mean ± standard error of the mean. Means are not significantly different between species (*t*-test, $P > 0.05$).

increments) at constant quantal irradiance, or irradiance was varied with neutral density filters, between 6.8×10^6 and 4.3×10^{10} photons·s^{-1}·cm^{-2}, at constant wavelength. The stimulus duration was controlled by a Uniblitz electronic shutter at 0.5 s for all trials. Individual colonies were allowed to dark adapt for a minimum of one hour before testing. In preliminary trials to determine the optimum interstimulus period, colonies produced less consistent responses (flash strength and number) to interstimulus periods of less than 3 min. Increasing the interstimulus interval beyond 5 min made no further improvement in response uniformity. Consequently, stimuli were delivered every 4 to 5 min, with the colony remaining undisturbed in the light-tight sphere during the interstimulus periods. The temperature of the seawater gradually increased from 12 to approximately 18°C during an experimental session, but no change in excitability was observed. Colonies produced few flashes in the absence of applied stimuli.

Stimulus irradiance was calibrated with a radiometer (United Detector Technology, Model S370) equipped with a silicon photodiode detector and a 180° cosine diffuser in the test specimen position. The presence of the stimulus baffle created a uniform diffuse stimulus; therefore, the radiant energy arriving at the surface of the sphere, as measured with the silicon photodiode cosine collector, is a measure of spherical irradiance. Because the pyrosome tissue is very clear, the zooid light receptor receives input from all directions, so the measured irradiance was multiplied by four to convert to scalar irradiance which is the energy per area arriving at a point from all directions about the point (Tyler and Preisendorfer, 1962). Stimulus irradiance was finally converted into quantal units, as (1) the number of photons may be more important than total energy in stimulating pyrosomes to produce light, and (2) to aid in comparisons with bioluminescence measurements.

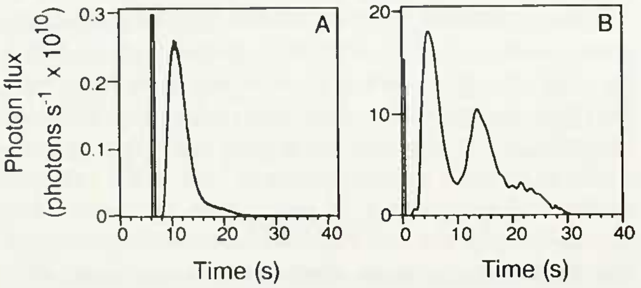

Figure 1. Varying luminescent responses of pyrosome colonies to photic stimuli at 475 nm, measured in an integrating sphere. The first flash is the stimulus artifact; it is followed by the bioluminescent response after a brief latency. The height of the stimulus flash does not represent the true intensity of the stimulus, due to detector saturation. (A) A simple response from *Pyrosomella verticillata*, due to simultaneous zooid light production. (B) A complex response from *Pyrosoma atlanticum* with two distinct peaks of luminescence.

Measured flash characteristics were:

(a) Latency—time from stimulus onset to flash onset;

(b) Rise time—time from flash onset to maximum photon flux of the flash;

(c) 98% response duration—time from flash onset to when photon flux has declined to 2% of maximum;

(d) Maximum flux—maximum flash intensity;

(e) Quantum emission—total integrated photons emitted over 98% response duration; and

(f) Mean emission—average integrated photons per second emitted during 98% response duration.

Images were intensified with an ISIT (Dage) low light level video camera with a 105 mm Nikon f/4 lens. Specimens were placed in Plexiglas chambers and enclosed in a light-tight container with white reflective internal surfaces. A photon counting photomultiplier system viewed the interior of the box, permitting simultaneous recording of relative flash kinetics and intensified video images. In some cases, specimens were examined with a dissecting microscope, with the ISIT camera recording the image through the photographic tube. Stimuli identical to those described above were used to elicit bioluminescence.

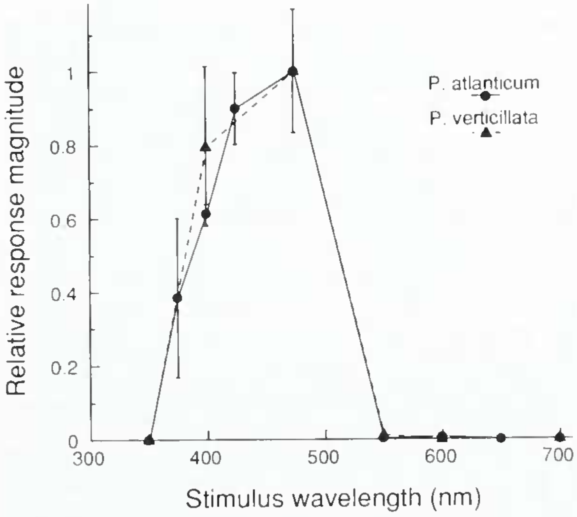

Figure 2. Normalized spectral responsivity of *Pyrosoma atlanticum* and *Pyrosomella verticillata*. Mean relative response magnitude is plotted as a function of stimulus wavelength; error bars represent standard errors of the mean. The stimulus scalar irradiance (500-ms pulse) for *P. atlanticum* was 1.5×10^9 photons · s^{-1} · cm^{-2}, and 7.1×10^8 photons · s^{-1} · cm^{-2} for *P. verticillata*. Peak response for both was at approximately 475 nm. Data fit a quadratic regression for both species, according to the equation $y = -19.07 + 0.0887x - (9.82 \times 10^{-5})x^2$, $r^2 = 0.98$ for *P. atlanticum*, and $y = -8.34 + 0.039x - (4.32 \times 10^{-5})x^2$, $r^2 = 0.98$ for *P. verticillata*. n = 4 colonies of each species.

The resulting MCA recorded waveform was stored and analyzed as previously described, while the video images were viewed at slow speed to analyze the propagation of signals. Video images were enhanced with a Megavision 1024XM image-analysis system for final presentation. The relative light emission of individual zooids was also examined with the image analysis system. In this analysis, the gray scale of the luminescent signal indicated the relative flash intensity of the region measured. This analysis was performed only on: (1) data collected with the ISIT video camera set to the manual gain setting, and (2) data not saturating the gray scale levels.

Results

Photic stimulation

In response to light stimuli, colonies often produced 25 to 30 flashes over approximately a 2-h period. Characteristic flashes had long latencies and durations and large quantum emissions (Table I). Flash latency and rise time were much less variable than quantum emission. Kinetic values for the two species (Table I) were not significantly different (ANOVA, $P > 0.05$). Light emission was independent of the colony surface area.

Colonies responded to spatially diffuse photic stimulation with varying flash displays, ranging from the most commonly observed simple flash (Fig. 1A), in which the responding zooids react approximately simultaneously, to more complex emission patterns (Fig. 1B). Such patterns may result from a variable latency in response to the initial stimulus, or to zooid reexcitation after a refractory period.

The spectral responsivity curves for both *P. atlanticum* and *P. verticillata* lay between 400 and 550 nm, and are described by a quadratic regression (Fig. 2). The spectral responsivity maxima were approximately 475 nm.

The half-response constant of photic excitation was determined by exposing specimens to between three and eight stimuli of identical scalar irradiance. The percentage of stimuli eliciting a response to 475 nm, regardless of magnitude, was plotted as a function of stimulus scalar irradiance (Fig. 3). Most stimuli elicited a response in either 0 or 100% of trials, except in a narrow range of irradiances. Three wavelengths were examined (graphs not shown for 400 nm and 600 nm), with similar response patterns observed.

Investigations of single visual receptor cells in insects produce similar results (Laughlin and Hardie, 1978; Hardie, 1979). In insects, the intensity response function follows the form

$$V/V_{max} = \mu I/(\mu I + 1), \qquad (1)$$

where I is the stimulus intensity, V is the response amplitude, V_{max} is the maximum response amplitude, and

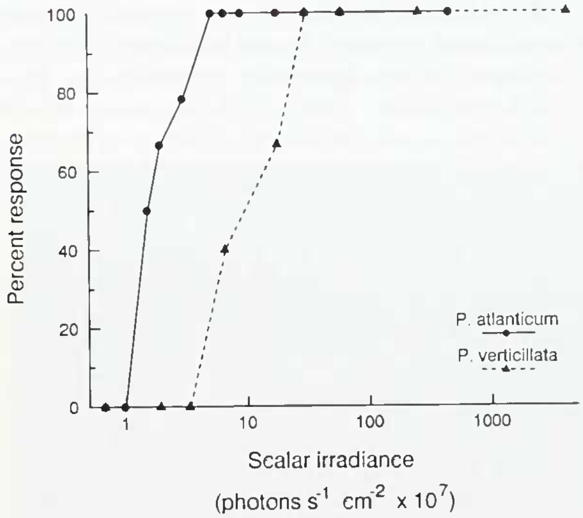

Figure 3. Bioluminescent response to 475 nm photic stimulation. The percentage of stimuli that elicited a response is shown as a function of the log of stimulus scalar irradiance. Data are grouped for all specimens. Calculated half-response constants are shown in Table II. Responses between 0 and 100% fit the linear regression $y = 10.13 + (1.9 \times 10^{-6})x$, $r^2 = 0.73$ for *Pyrosoma atlanticum*, and $y = 4.12 + (3.2 \times 10^{-7})x$, $r^2 = 0.91$ for *Pyrosomella verticillata*. n = 3 colonies of each species.

μ is the sensitivity parameter and equals the reciprocal of the intensity required to produce a response 50% of maximum (Laughlin, 1975). The half-response constant is the level at which the slope of the V/log I curve is maximal, and is defined in this study as the threshold level of photic stimulation that produces a bioluminescent response. Using formula (1), the half-response constant for *P. atlanticum* to 475 nm was significantly lower than for *P. verticillata* (Table II; *t*-test of slopes and points of linear regressions, $P < 0.05$). Half-response constants to 400 and 600 nm stimuli were not significantly different (Table II). The half-response constant to photic stimulation was independent of the overall colony length.

Colony quantum emission and flash duration were proportional to stimulus irradiance. Relative quantum emission (Fig. 4A) and 98% flash duration (Fig. 4B) varied logarithmically with stimulus scalar irradiance. The maximum and mean emissions, though, did not vary consistently with the stimulus scalar irradiance. Thus the scalar irradiance effect on flash duration may account for the change in quantum emission. To clarify these relationships, the relative light emission of individual zooids was examined with the image analysis system. Individual zooids in unvarying orientation during flash events elicited by photic, mechanical, and electrical stimuli had remarkably constant flash intensities. The quantum emission per zooid varied by less than 10% among flashes, independent of the colony quantum emission. Differences between flashes were often less than 2%. Light emission of zooids began to decrease only after about 10–15 flashes spaced about 30 s apart. Image analysis also revealed that more intense or repeated stimuli caused increasing numbers of zooids to respond asynchronously, thus increasing the total flash duration of the colony. This relationship between stimulus scalar irradiance and the fraction of zooids responding is evidence for a variation in the half-response constant among zooids. Thus, the dependence between quantum emission and stimulus scalar irradiance is due to the asynchronous triggering of greater numbers of zooids, leading to longer colonial flash durations.

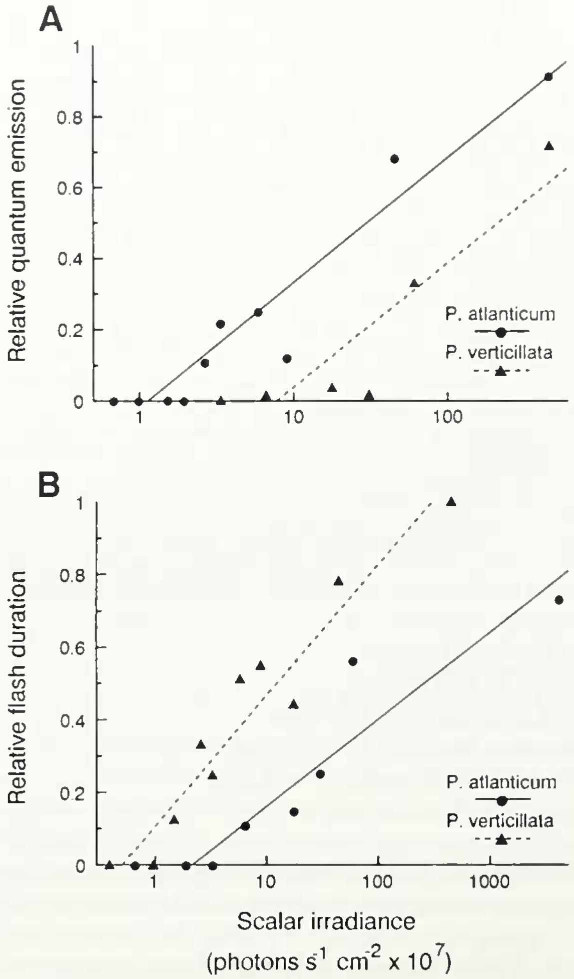

Figure 4. Quantum emission and flash duration elicited by 475 nm photic stimuli of varying scalar irradiance. The increase in quantum emission with the increase in stimulus scalar irradiance was due to greater flash durations caused by the asynchronous triggering of zooids. (A) Relative quantum emission of colonies from 2 to 7 cm length. The slope of the logarithmic regression for *Pyrosoma atlanticum* is 0.15 ($r^2 = 0.92$); that for *Pyrosomella verticillata* is also 0.15 ($r^2 = 0.83$). There is no significant difference between the regression slopes or elevations (*t*-test, $P > 0.05$). (B) Relative 98% flash durations. The slope of the logarithmic regression for *P. atlanticum* is 0.10 ($r^2 = 0.86$); that for *P. verticillata* is 0.16 ($r^2 = 0.91$). These slopes are significantly different (*t*-test, $P < 0.05$).

Table II

Half-response constants (photons · s^{-1} · cm^{-2}*) to photic stimulation (*V_{max} *= 50% response) calculated from the V/log I function of Laughlin, 1975*

	Stimulus wavelength (nm)		
	400	475[a]	600
Pyrosoma atlanticum	1.1×10^8	1.1×10^7	9.7×10^8
Pyrosomella verticillata	2.6×10^8	9.3×10^7	3.3×10^9

[a] Slopes and points on linear regression significantly different between species (*t*-test, $P < 0.05$). Variances are not significantly different (F test, $P > 0.05$).

Image intensification revealed strikingly different colonial patterns of luminescence in the two species investigated. In *P. atlanticum*, small and large zooids are intermixed throughout the colony. This pattern is evident as an irregular pattern of small and large luminescent sources distributed over the colony surface (Fig. 5A). The zooid light organs lie close together, often producing apparent single points of light, which are resolved under higher magnification into pairs of luminous sources. *P. verticillata*, in contrast, possesses zooids of uniform size in distinct rows, with a wider spacing between the pair of light organs in each zooid. This results in uniform rows of luminescent sources over the colony surface (Fig. 5B). These obvious differences in light patterns, rooted in the colony morphology, make the two species easily distinguishable by their luminescent patterns.

Bioluminescence propagation

ISIT video records of events caused by a single mechanical stimulus revealed that luminescence begins from the point of stimulation and slowly spreads across the colony in all directions, at an overall rate of 2.1–4.1 $mm \cdot s^{-1}$, at temperatures of 12 to 16°C (Fig. 6). The light usually travels across the colony by saltatory conduction, the nodes being either single zooids or groups of zooids 0.5 to 1.5 cm apart, with a latency between nodes of about 3 s. Zooids between the responsive regions begin to luminesce after being bypassed, while the wave continues to the next responsive site. This disjointed wave of bioluminescence is characteristic of both species examined.

Electrical stimulation

Single electrical stimuli produced flashes of simple shape, with few irregularities in the waveform (Fig. 7A; Table III). Temporal summation of luminescence was induced by electrical pulses at 0.5 and 1 Hz (Fig. 7B). Video analysis revealed that this summation was due to increasing recruitment of zooids with successive stimuli and, to a lesser extent, an increase in zooid light emission. In trials with constant stimulus rates of 5 to 50 Hz, luminescence was produced initially, and was often followed by a series of shorter, repeating flashes (Fig. 7C) with an average interflash period of 18 s. In a few trials, however, light was elicited at a similar initial rate, but no repetitive flashing pattern was observed (Fig. 7D). Multiple stimulation elicited a significantly larger response duration, maximum flux, and mean emission than single pulses (*t*-test, $P < 0.05$; Table III). Rise time, quantum emission, and flash duration in response to electrical stimuli were significantly different from these parameters for photic excitation (Tukey test, $P < 0.05$). The maximum flux and quantum emission were again independent of colony surface area.

Mechanical stimulation

Repetitive mechanical stimulation induced a significantly greater light emission than any other stimulus method employed in this study (Tukey test, $P < 0.05$; Table IV). The total duration of light emission in all cases exceeded the 200-s collection period, with a mean flash duration of 59 s. Within this period a repeating flash pattern, with similar kinetics to that for electrical stimuli, was observed (Fig. 8A).

Many colonies also produced bioluminescent flashes with simple kinetics (Fig. 8B) in the absence of any obvious external stimuli except ship movement (Table IV). Bioluminescent events of this type occurred randomly during the interstimulus resting periods and were thus easily separated from flashes elicited by photic or other stimuli. Unlike photically or electrically stimulated organisms, the light emission for mechanically induced bioluminescence was directly related to the colony surface area (Fig. 9).

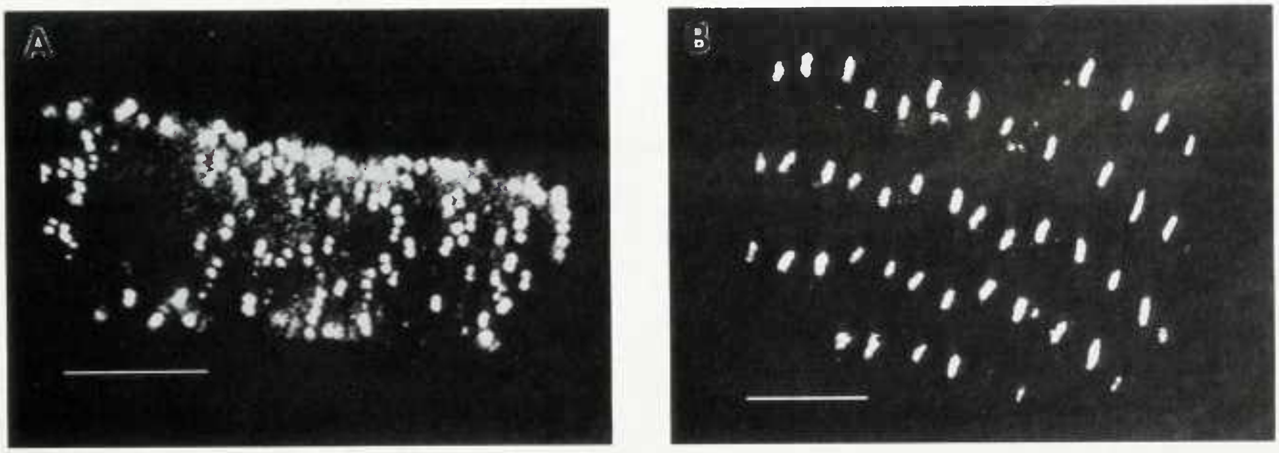

Figure 5. Single ISIT video frames of patterns of luminescence in response to photic stimuli. Arrangement of luminous sources is based upon colony morphology. (A) *Pyrosoma atlanticum*, showing an irregular pattern of luminescent sources. Bar = 1 cm. (B) *Pyrosomella verticillata*, exhibiting uniform distribution of luminescent sources. Bar = 0.5 cm.

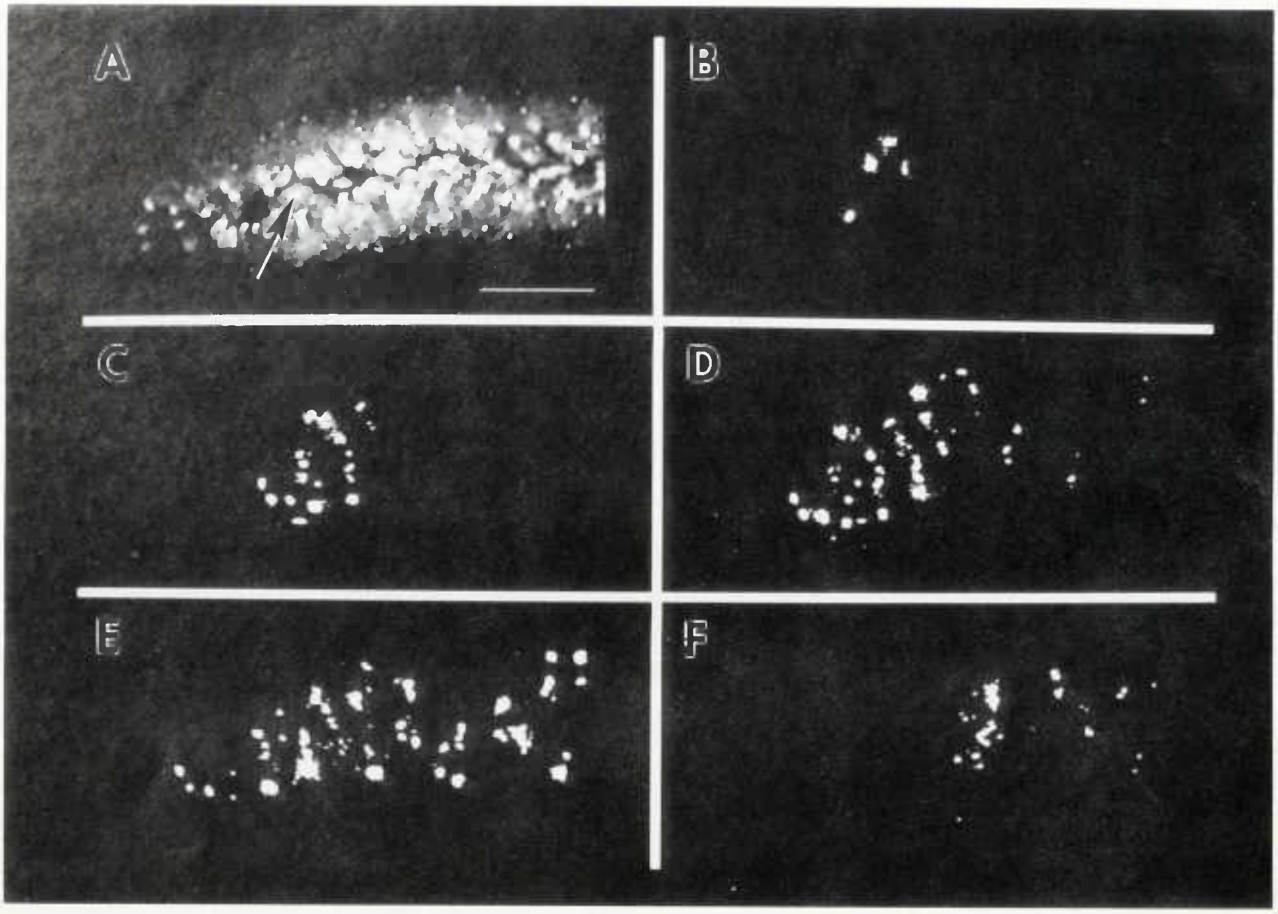

Figure 6. A bioluminescent wave traveling across *Pyrosoma atlanticum*. The colony is oriented in the same position in (A) through (F). (A) Image of the colony with red illumination. Arrow indicates the point of mechanical stimulation. Bar = 1 cm. (B) The flash begins at the point of stimulation (time = 0 s). (C) After 3 s the luminescent response has spread to other nearby zooids. Progressive bidirectional conduction of the light wave across the colony at (D) 6 s and (E) 9 s after the beginning of the response. (F) Decay in intensity of the response (time = 15 s).

Therefore, most zooids in the colony probably responded to mechanical stimuli, in contrast to responses to the other stimulation methods.

Discussion

Bioluminescence of pyrosome colonies begins at the location of the stimulation, and slowly propagates by a photic, saltatory conduction process in all directions. The photic propagation of light along the colony is supported by several pieces of evidence. First, the similarity between our action spectrum and the luminescent emission spectrum (Swift *et al.*, 1977; Widder *et al.*, 1983) indicates that the colony responds to, and emits, the same wavelengths of light. Second, light from one zooid is also easily able to surpass the half-response constant of other nearby zooids. According to Allard's law

$$E_x = Ie^{-cx}/x^2, \qquad (2)$$

light of intensity I will be attenuated in the sea, at a distance x, to an irradiance E, where c is the light attenuation coefficient (Jerlov, 1968). Using formula (2) and an attenuation coefficient of 0.05 for Type I Hawaiian waters (Jerlov, 1968), the approximate luminescent output of an individual *P. verticillata* zooid, calculated from the colony maximum flux and total number of zooids, decays to the colonial photic half-response constant at 2.6 m, assuming no absorption or scattering due to pigments in the colony. Finally, the variation among zooids in their half-response constants reflects their ability to respond independently to light.

Progression of the colonial luminescent wave is not dependent upon intact connections between the zooids, clearly indicating that the wave propagates by a photic process (Burghause, 1914). Mackie and Bone (1978) photically stimulated the tetrazooid of *P. atlanticum* and calculated that, if the luminescent wave were propagated

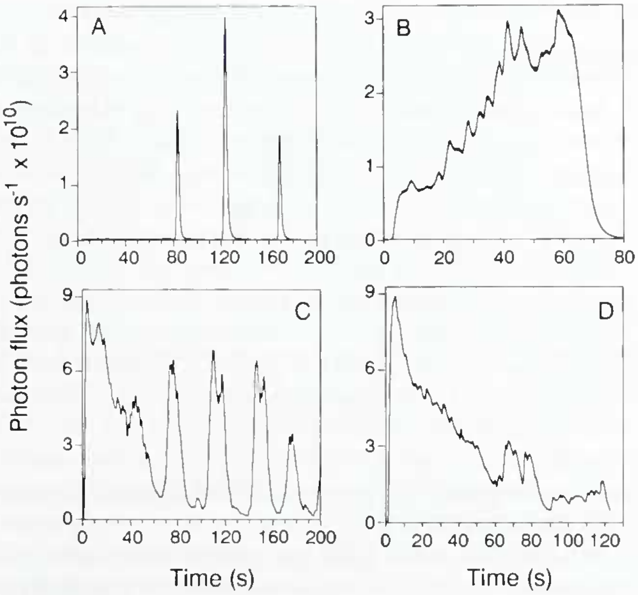

Figure 7. Examples of electrically induced bioluminescence of *Pyrosomella verticillata*. Fifty-volt, 5-ms duration pulses were applied to the medium. (A) Single stimulus delivered at 80 s, 120 s, and 165 s; each produced a simple response. (B) Stimuli delivered at 1 Hz for 60 s, starting at time = 0, showing temporal summation. (C) Constant 5 Hz stimulation, yielding a regular flashing pattern, perhaps due to an 18-s refractory period characteristic of the zooids. (D) Constant 10 Hz stimulation, eliciting light at a similar initial rate to (C), but without a regular flashing pattern.

by the serial excitation of zooids, the colony propagation velocity, taking into account the response delay and the distance between zooids, would be 2.0–4.0 mm · s^{-1}. This value is remarkably similar to the observed saltatory propagation rate of 2.1–4.1 mm · s^{-1} found in this study of mature colonies. Mackie and Bone also found no nerves or gap junctions associated with the light organ, and deemed it unlikely that conducting epithelia could propagate this activity, because the normal rate of epithelial conduction in tunicates is about 20 cm · s^{-1}. In addition, no specific cellular depolarizations were associated with flashing, and the mantle epithelium, to which the light organ is attached, is not a conducting type in the taxonomically similar ascidians. Luminescent waves typically propagate in the colonial coelenterate *Renilla* at 6–10 cm · s^{-1} (Nicol, 1955; Morin and Cooke, 1971), and 20–50 cm · s^{-1} in hydrozoa (Widder *et al.*, 1989). These high propagation rates are enabled by the underlying nervous tissue. These data indicate that the luminous wave is propagated by a photic chain reaction.

The mechanism underlying the saltatory conduction of luminescence may derive in part from the different photic half-response constants of the zooids. This is a reasonable finding, as each zooid contains its own light detection and production organs (Bone and Mackie, 1982; Mackie, 1986). The zooids thus seem to act independently of one another, rather than as a single, integrated colonial receptor.

The variation in colony half-response constant may be due in part to colony size. Larger colonies necessarily absorbed a larger number of photons entering the sphere; less light is therefore incident per zooid for a given number of photons. Thus, half-response constants may have been artificially elevated for larger colonies.

Continuous excitation of a colony often produced a rhythmic colonial flashing pattern, characterized by alternating periods of high and low light emission. Although some light is produced between flashes under these conditions, the majority of zooids are quiescent. Zooids thus appear to possess a refractory period of about 18-s duration, during which their half-response constant is greater than the stimulation that they receive.

The existence of a refractory period is further supported by the quenching of a single wave of luminescence elicited by a local mechanical stimulus. In long colonies, light

Table III

Kinetics and intensities of Pyrosomella verticillata *flashes stimulated with single or repetitive electrical pulses. 50-V, 5-ms pulses were applied to the medium. Values represent the mean ± standard error of the mean*

Stimulus type	Latency (s)	Rise time (s)	98% Duration (s)	Maximum flux (photons · s^{-1})	Mean emission (photons · s^{-1})	Quantum emission (photons · flash^{-1})
Single (n = 4)	5.6	2.1 ± 0.3	8.8 ± 1.6^{a}	2.3×10^{10} $\pm 6.6 \times 10^{9}$	$6.1 \times 10^{9\,a}$ $\pm 1.3 \times 10^{9}$	5.9×10^{10} $\pm 2.1 \times 10^{10}$
Multiple (n = 11)	3.4 ± 1.2	10.1 ± 2.7	78.3 ± 21.8^{a,b}	6.6×10^{10} $\pm 1.4 \times 10^{10}$	$2.7 \times 10^{10\,a}$ $\pm 5.7 \times 10^{9}$	3.7×10^{12} $\pm 1.5 \times 10^{12}$

[a] Means are significantly different (*t*-test, $P < 0.05$).

[b] Mean underestimates the actual value, because some responses persisted beyond the data collection period.

Table IV

Kinetics and intensities of mechanically stimulated pyrosome flashes. Values represent the mean ± standard error

Species	Stimulus type	Rise time (s)	98% Duration (s)	Maximum flux (photons · s^{-1})	Mean emission (photons · s^{-1})	Quantum emission (photons · $flash^{-1}$)
Pyrosoma atlanticum	Constant prodding (n = 9)	20.0 ± 1.6	59.2 ± 14.6	3.3×10^{12} [a] ± 3.1×10^{11}	6.6×10^{11} ± 6.0×10^{11}	2.3×10^{13} ± 1.9×10^{13}
Pyrosome sp.	Ship movement (n = 6)	8.3 ± 3.0	25.2 ± 6.7	7.5×10^{11} [a] ± 6.8×10^{11}	1.7×10^{11} ± 1.3×10^{11}	3.8×10^{12} ± 2.7×10^{12}

[a] Means are significantly different between stimulus methods (*t*-test, $P < 0.05$).

from the stimulus area was extinguished when the wave of light was at the far end of the colony. As a zooid's maximum flux decays to the colonial half-response constant at about 2.6 m, the wave of light should be able to reexcite the previously responsive parts of the colony. Reexcitation, however, is rarely observed, with a colonial response to a single stimulus usually subsiding after one pass across the colony.

The bioluminescent response to light flashes implies several uses of light for the colony. Using Allard's law for Hawaiian waters, the maximum flux for *P. atlanticum* (mechanical stimuli) would decline to the photic half-response constant at 78 m. Few zooids in a colony would respond, however, to this dim level of luminescence. The maximum quantum emission observed to photic stimuli in this study could be induced at a distance of 17 m, indicating that flash entrainment among colonies may occur at large distances. Roe *et al.* (1987) report a maximum of 85 colonies per 10,000 m^3 at 800 m in the Atlantic near the Canary Islands. The model of closest packing of equal spheres allows for one colony every 5.5 m, indicating that, in some areas of the ocean, flash entrainment among colonies may produce widespread displays (Mackie and Mills, 1983).

Flash entrainment may also occur interspecifically. Most other planktonic organisms produce bioluminescence of the same wavelengths as that of pyrosomes (Young, 1981; Herring, 1983; Widder *et al.*, 1983; Latz *et al.*, 1988); this light could also stimulate pyrosome luminescence if it were of sufficient intensity. For example, a flash from the common copepod *Pleuromamma xiphias* in Hawaiian waters would be sufficient to elicit luminescence in *P. atlanticum* at 14 m (Latz *et al.*, 1987).

Luminescence is often produced in response to a disturbance by a predator, and is conventionally thought to confer protection by startling or blinding the predator, or by attracting a secondary predator (David and Conover, 1961; Morin, 1983; Young, 1983; Buskey and Swift, 1983,

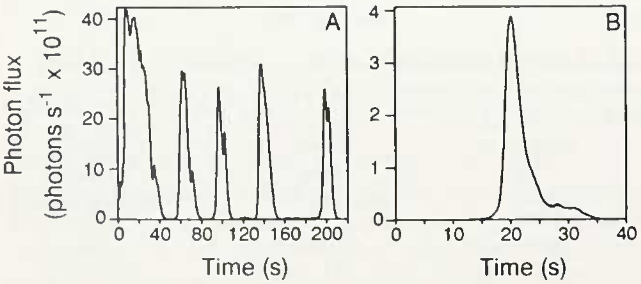

Figure 8. Examples of luminescence by *Pyrosoma atlanticum* induced by mechanical stimulation. (A) Periodic stimuli (1–2 Hz) produced a regular flashing pattern similar to that caused by repetitive electrical stimulation. Individual flashes within the pattern averaged 59-s duration. (B) A simple flash presumably induced by ship motion.

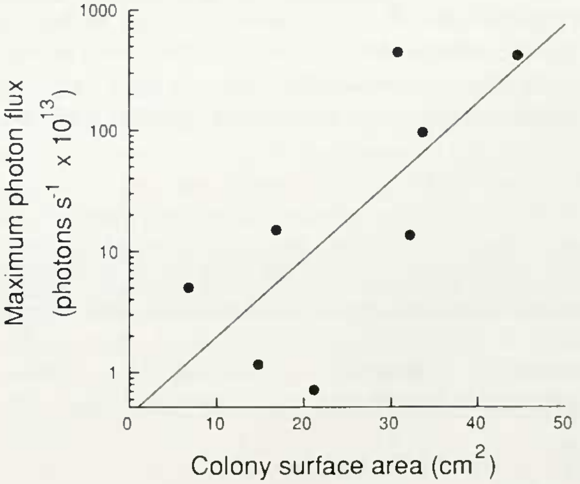

Figure 9. Maximum bioluminescence production as a function of colony surface area, including both species. Luminescence was induced by mechanical stimulation, as this method produced the greatest light emission and thus most closely approximated a colonial response of all zooids. The slope of the exponential regression is 0.15 ($r^2 = 0.55$).

1985). Pyrosomes also display an additional set of behaviors in response to photic stimuli; zooids close their oral openings, arrest their cilia (resulting in the suspension of locomotion), and produce luminescence (Mackie and Bone, 1978; Mackie, 1986). Being negatively buoyant, the colony would sink into deeper layers until the recommencement of ciliary action, thus perhaps evading predation by leaving a depth of high predator density (Mackie and Bone, 1978). Flashing may serve as a means of communication between distant zooids or colonies, enabling them to close protectively and sink before oncoming harmful stimuli can arrive. Photically stimulated bioluminescence may also discourage predators by making the colony, or a group of adjacent colonies, loom up out of darkness, perhaps giving the impression of a very large source that should not be trifled with. Simultaneous luminescence from many spatially separated sources might also distract a predator from a single target, analogous to the simultaneous displays of fish schools in the photic zone (Radakov, 1973; Morin, 1983).

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Circadian Rhythmicity of the Crustacean Hyperglycemic Hormone (CHH) in the Hemolymph of the Crayfish

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Abstract. The crustacean hyperglycemic hormone (CHH) is involved in the regulation of endogenous blood glucose metabolism. In this paper we describe the daily rhythmicity in the blood glucose and the blood CHH content of the crayfish *Orconectes limosus*. Both blood CHH and blood glucose levels increase during the first hours after the beginning of darkness. The bioactivity of released CHH is far higher than that of CHH stored in the sinus gland. Moreover, the released hyperglycemic material shows an affinity for high molecular weight proteins in the hemolymph. Preliminary results suggest that subunits of hemocyanin may act as potential carrier-proteins for bioactive CHH.

Introduction

The neuroendocrine system producing the crustacean hyperglycemic hormone (CHH) of decapod crustaceans forms part of the medulla terminalis ganglionic X-organ (MTGX). The MTGX lies at the outer edge of the medulla terminalis—the most proximal optic ganglion in the eyestalk—and contains several hundred neuroendocrine cells. In crayfish, such as *Astacus leptodactylus* and *Orconectes limosus*, about 35 to 40 CHH-producing cells form a distinct group located latero-ventrally on the MTGX. Neurosecretory granules containing CHH are transported via a tract that leads across the neuropil of the medulla terminalis to a neurohemal region, the sinus gland. Immunocytochemical and morphometric research indicates that about 40% of the sinus gland axon terminals are filled with neurosecretory granules containing CHH, which is released into the hemolymph by exocytosis. The hyperglycemic hormone in the blood regulates blood sugar levels to meet physiologically required metabolic energy needs (Strolenberg and Van Herp, 1977; Strolenberg *et al.*, 1977; Van Herp and Van Buggenum, 1979; Gorgels-Kallen and Van Herp, 1981; Gorgels-Kallen *et al.*, 1982; for a review see Kleinholz, 1985).

The glucose level in the hemolymph of decapod crustaceans reveals a day/night rhythmicity, characterized by a low basal level during the light period, and a peak in glucose content appearing several hours after the onset of darkness (Hamann, 1974; Strolenberg, 1979; Reddy *et al.*, 1981). The basal level during the day, as well as the height and duration of the nocturnal peak, are species-dependent and are affected by seasonal influences (unpub. obs.) as well as physiological events, such as molting (Kallen, 1985). Physiological research in crayfish strongly indicates an endogenous circadian blood glucose rhythm entrained by the light/dark schedule (Kallen *et al.*, 1988).

In previous studies, we investigated the secretory dynamics of the CHH-system of *Astacus leptodactylus*. Immunocytochemical staining combined with morphometric analyses at the light and electron microscopic level revealed a daily rhythmicity in the synthetic activity of the perikarya, the transport of CHH-material to the sinus gland, and the release of CHH into the hemolymph (Gorgels-Kallen and Voorter, 1984, 1985). In this study, we report and discuss the immunochemical detection of circulating bioactive CHH in the hemolymph during a 24-h period. We present a preliminary molecular characterization of the bioactive CHH present in the blood and

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compare it to the molecular form of the hormone stored in the sinus gland[1].

Materials and Methods

Animals

Crayfish (*Orconectes limosus*) were obtained from a commercial fisherman, who had collected them in the river Meuse. In the laboratory, the animals were kept in running tap water (13–15°C) and fed weekly with ground meat or fish. Experiments were performed with adult intermolt female and male crayfish of equal size and weight (about 20 g). The animals were kept under constant light/dark conditions (LD 12:12; light on 8:00 a.m.).

Antisera

Immunochemical quantification was carried out with an enzyme-linked immunosorbent assay (ELISA); the double antibody-sandwich (DAS) method was followed. The first antiserum was a polyclonal anti-CHH serum raised in rabbits against a biologically active CHH preparation from sinus glands of the crayfish *Astacus leptodactylus*. Details about the purification of the antigen, the production of antiserum, and tests of specificity have been described previously (Gorgels-Kallen and Van Herp, 1981). The second antiserum was a polyclonal anti-CHH serum raised in mice against a biologically active CHH preparation from sinus glands of the crayfish *Orconectes limosus*. The production of this antiserum is described below.

The CHH was isolated from dissected sinus glands that had been collected in 0.1 *N* HCl, homogenized, and extracted overnight in 0.1 *N* HCl. The extracts were centrifuged and the supernatants lyophilized. This crude extract was further purified by preparative polyacrylamide gel electrophoresis (according to Davis, 1964), followed by gel filtration on a calibrated Sephadex G-50 superfine (sf) column. Quantitative protein determination was carried out by absorption at 280 and 220 nm. The purified fractions were tested in a bioassay (according to Leuven *et al.*, 1982), and immunoreactivity was tested by a direct ELISA using the anti-*Astacus*-CHH serum mentioned above. The resulting purified material was a biologically and immunologically active peptide with a MW of around 6500 Da, which is in agreement with former results (*e.g.*, Keller, 1977; Kallen *et al.*, 1986). Female Swiss mice received a first intraperitoneal injection comprising 5 μg purified CHH material buffered with 0.03 *M* ammonium acetate, pH 8.5, and emulsified in Freund's complete adjuvant (1:1). After 10 and 17 days, booster injections containing, respectively, 3 and 2 μg in buffer and emulsified 1:1 in Freund's incomplete adjuvant, were given intraperitoneally. After 27 days, blood was collected by heart puncture. The specificity of the sampled mouse serum was tested in a dilution series by a direct ELISA, carried out in microtiter wells coated with 10 ng of the purified CHH material. Immunoreactivity of the antiserum disappeared after absorption with purified CHH and crude sinus gland extract. Pre-immune serum was immunochemically negative with purified CHH and crude sinus gland extract.

Hemolymph sampling

Hemolymph was sampled over a 24-h period; the sample times were chosen based on former results (Gorgels-Kallen and Voorter, 1985). During this sampling period, the animals were kept in a series of tanks, each tank containing five crayfish. At each sample time, the hemolymph from one group of five crayfish was collected, as described by Gorgels-Kallen and Voorter (1985) for the crayfish *Astacus leptodactylus*. From each animal, two 50-μl samples of hemolymph were aspirated into a calibrated capillary pipet that had been inserted between coxa and basis of the left cheliped. The first hemolymph sample was diluted immediately in an equal volume of buffer (0.1 *M* phosphate solution containing 0.01 *M* sodium citrate and 0.01 *M* EDTA, pH 7.5, to prevent clotting). This sample was used for ELISA. The duplicate sample was frozen immediately, and the blood glucose level was determined by the Gluco Quant Test Combination (Boehringer Mannheim GmbH). For each time point, we report the mean and standard error of the five determinations of CHH and glucose, respectively.

Immunochemical analysis of blood samples

The DAS-ELISA technique was carried out according to Voller *et al.* (1979). After pretreatment with 1% glutaraldehyde, microtiter plates (Nunc) were coated with 100 μl *Astacus*-CHH serum (rabbit serum: optimal dilution 1/1000 in 0.05 *M* sodium carbonate buffer, pH 9.4; incubation for 1 h at 37°C followed by incubation overnight at 4°C). After nine washes in ELISA-buffer (0.02 *M* phosphate buffer, pH 7.4, containing 0.05% Tween 20 and 0.9% NaCl), the wells were adsorbed with 100 μl bovine serum albumine (BSA) and 1% normal goat serum (NGS) dissolved in ELISA-buffer (30 min at room temperature). Each well was then incubated with 100 μl of the different hemolymph samples diluted 1:1 in ELISA-buffer (1 h at 37°C). One hundred microliters of anti-*Orconectes*-CHH serum (mouse serum: optimal dilution 1/200 in ELISA-buffer, 1.5 h at 37°C) was then added, followed by peroxidase-labeled rabbit-anti-mouse (RAM) serum containing 1% NGS (diluted 1/2000 in ELISA-buffer, 1 h at 37°C). The wells were washed, as described above, between each incubation. The enzymatic activity was initiated by the addition of orthophenyl-diamine acid (250 μl: 1 mg/ml in 0.1 *M* Mac-Ilvaine buffer, pH 5.2, containing 1 μl/ml 40% H_2O_2). The reaction was stopped by the addition of 250 μl 4 *N* H_2SO_4. The optical density was measured at 492 nm with an EAR-400 ELISA-reader (Austria Instruments). The detection range of the DAS-ELISA was determined by assaying a dilution series of purified CHH. The lower limit of detection is about 60 pg purified CHH (10–15 fmol).

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[1] Results were presented at the 14th Conference of European Comparative Endocrinologists, Salzburg (4–9 September 1988): Kallen, J. L., and Abrahamse, S. L. (1989).

Biochemical analyses of hemolymph samples

Hemolymph samples were analyzed by gel filtration on a calibrated Sephadex G-200 superfine (sf) column (diam: 1.5 cm; length: 45 cm) eluted with 0.1 *M* phosphate buffer pH 7.5 (containing 0.01 *M* sodium citrate and 0.01 *M* EDTA to prevent blood clotting). The flow rate was 5 ml/h, and 1-ml fractions were collected. For each analysis, 750 μl of hemolymph from one animal was sampled. The sample was taken at the start of the dark to coincide with the exocytosis, expected during that period, of CHH into the blood (Gorgels-Kallen and Voorter, 1985). After gel filtration, the immunological activity of each column fraction was tested by DAS-ELISA.

The hyperglycemic activity of each column fraction was also tested with a bioassay for hyperglycemia, as described by Leuven *et al.* (1982). As controls, animals were injected either with physiological saline (Van Harreveld solution), or with 5 μg bovine serum albumin (BSA) dissolved in saline. The latter control was performed to test the effect on glucose levels of injecting large amounts of high molecular weight proteins. The amount of BSA corresponded with the amount of protein (after Lowry *et al.*, 1951) in the injected blood fractions.

Crude hemolymph samples and fractions obtained after gel filtration were further studied using sodium dodecylsulphate-polyacrylamide-gel electrophoresis (SDS-PAGE), according to Laemmli (1970). The fractions to be analyzed were diluted in 0.01 *M* Tris/HCl buffer, pH 6.8, containing 10% glycerol and 1% SDS (crude hemolymph and gel fractions diluted respectively 1:20 and 1:10 in buffer). Each fraction contained 1.5–2 μg protein measured according to Lowry *et al.* (1951). Electrophoresis was carried out with a 3% stacking gel (0.1% SDS in 0.125 *M* Tris/HCl buffer, pH 6.8) and a 10% separating gel (0.1% SDS in 0.375 *M* Tris/HCl buffer, pH 8.8); a 0.05 *M* Tris/0.384 *M* glycine buffer containing 0.1% SDS, pH 8.3, was used. The gels were either stained with Coomassie Brilliant Blue R 250, or immunochemically analyzed by immunoblotting, performed with a Biorad trans blot cell and a Biorad 160/1.6 power supply. Proteins were electrophoretically blotted on nitrocellulose paper: overnight at 30 V, and then for 1 h at 60 V (Schleicher and Schüll BA 85; 0.45 μm); a 0.025 *M* Tris/HCl buffer, pH 8.3, containing 0.192 *M* glycine and 10% methanol was used. After electrophoresis, the nitrocellulose paper was stained immunochemically as follows. Blots were washed for 5 min in TBS (0.05 *M* Tris/HCl buffer, pH 7.4, containing 0.9% NaCl and 0.1% Tween 20). After washing, blots were blocked for 15 min with 3% BSA and 1% NGS in TBS (referred to as blocking solution: BS). They were then incubated for 1 h with anti-*Orconectes*-CHH (mouse serum, dilution 1/1000 in BS). After four washes in TBS, blots were incubated for 1 h in rabbit-anti-mouse peroxidase (RAM-PO: dilution 1/2000 in BS). After four washes in TBS, blots were stained with 3.3′-diaminobenzidine-4 HCl (DAB: 1 mg per 8 ml TBS, containing 0.3% H_2O_2). All incubations were performed at room temperature.

Results

Rhythmicity in the blood glucose and CHH level

During a 12 h L:12 h D period, the blood glucose level of the crayfish *Orconectes limosus* shows a clear rhythmicity (Fig. 1A). During the light period, a rather low basal level is observed, but during the first 3 to 4 h after onset of darkness, blood glucose levels increase by two- to fourfold. We measured 24-h blood glucose rhythms in animals four weeks after their arrival at the laboratory, and also in animals that had been kept under

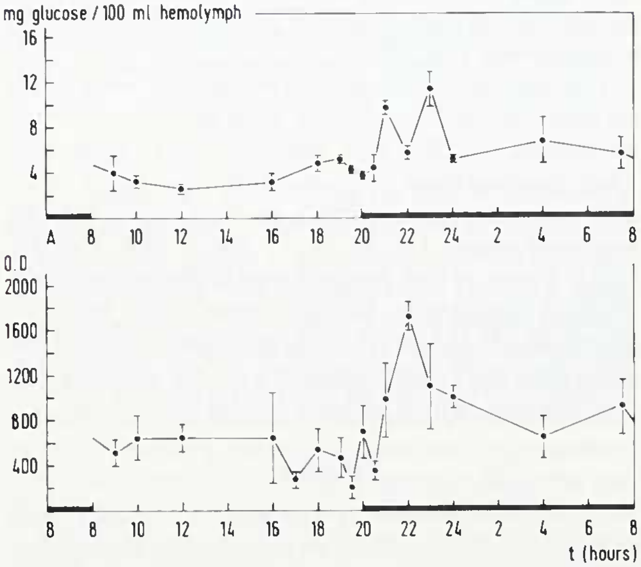

Figure 1. Hemolymph glucose and CHH levels during a 24-h period, determined for crayfish (*Orconectes limosus*) kept under constant 12 h light/12 h dark conditions; light on at 8:00 a.m. (a) Hemolymph glucose levels; means ± SEM; n = 5. (b) Hemolymph CHH levels in the same samples; means ± SEM; n = 5.

experimental conditions for up to a year. The majority of measured 24-h blood glucose cycles show two successive peaks. Hamann (1974) reported 24-h blood glucose rhythms for *Orconectes* with a single glucose increase at the beginning of the dark period. However, he took fewer blood samples at 4-h time intervals. Our results show that the two glucose peaks appear within the first 4 h of darkness. They are only detectable when blood samples are taken more frequently. The physiological significance of this phenomenon is not yet clear to us.

Figure 1B presents the levels of immunodetectable CHH in the duplicate hemolymph samples, measured in a DAS-ELISA. These results show a comparable pattern. The CHH positive fraction in the hemolymph remains on about the same level during the light period and then, during the first 3 h after the onset of darkness, increases steeply threefold.

Biochemical analysis of hemolymph

Analysis of hemolymph samples on a calibrated Sephadex G-200 sf column revealed a broad absorption peak between fractions 25 and 38 (Fig. 2A). Thus, most of the detectable proteins in the hemolymph vary in molecular weight between 60 and 150 kDa. CHH immunoreactivity was found in fractions 29 to 35, with a maximum in fraction 32 (Fig. 2B). When this fraction, obtained from 750 μl of hemolymph, was injected into five crayfish, it produced a level of hyperglycemic activity higher than that of any other fraction. The activity was comparable to the blood glucose increase induced by the injection of 0.5 sinus gland equivalents per animal. Injection of a comparable dose of high molecular protein (BSA) caused no hyperglycemia (Fig. 2C).

The immunochemically and biologically most active gel filtration fractions (fraction number 31, 32, 33) were investigated on SDS-PAGE, followed by immunoblotting. These fractions show very similar patterns (Fig. 3). They all contain immunodetectable proteins with molecular weights of around 150, 80, 74, 72, and 56 kDa. This pattern is similar to that obtained after SDS-electrophoresis of crude hemolymph. Electrophoresis of the immunochemically (ELISA) and bioactively negative gel fractions shows only weak immunostaining for the regions corresponding to a MW of 150 and 74 kDa (Fig. 3A, B).

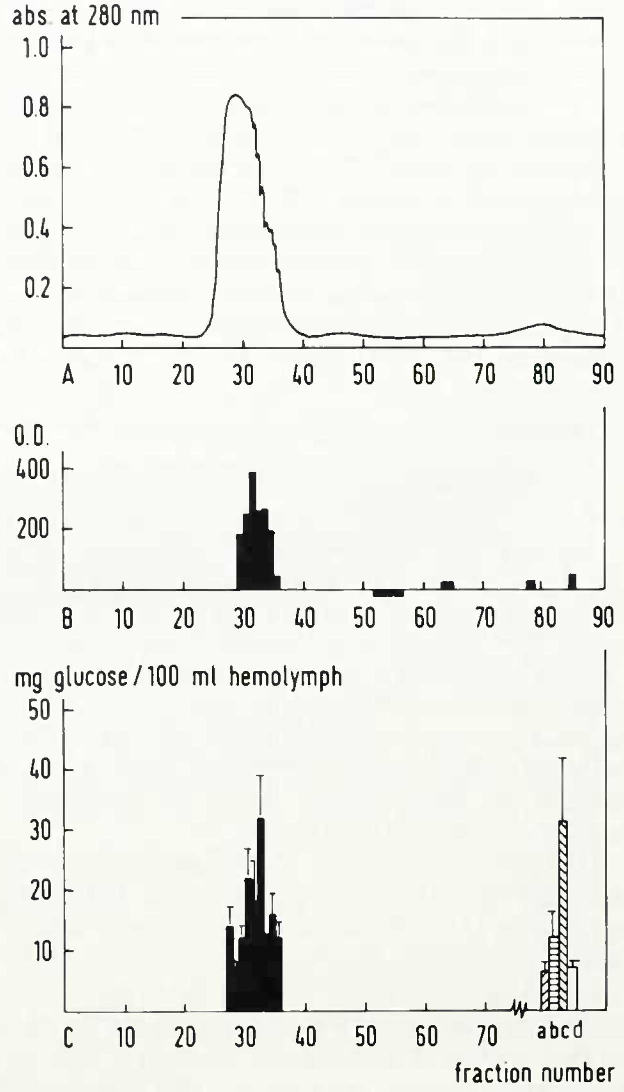

Figure 2. Gel filtration of the hemolymph of the crayfish (*Orconectes limosus*). (a) Gel filtration of 750 μl hemolymph on a Sephadex G-200 sf column. (b) Immunological response determined by a DAS-ELISA of the column fractions. (c) Hyperglycemic response after injecting the column fractions; means ± SEM; n = 5. a = pre-injection value; b = control injection with elution buffer; c = control injection of 0.5 sinus gland equivalents per animal; d = control injection of 5 μg BSA per animal.

Discussion

This paper demonstrates the diurnal rhythmicity in the levels of glucose and CHH in the blood of the crayfish, *Orconectes limosus*. The rhythm in blood glucose emerges as a low basal level during the day and an increase during the night—a day/night pattern comparable to that described previously for *Astacus leptodactylus* (Strolenberg and Van Herp, 1977; Gorgels-Kallen and Voorter, 1985). In *Astacus*, however, the blood glucose peak appears 3 to 4 h after the onset of darkness and remains elevated for most of the night, whereas in *Orconectes* it only increases during the first 3 h of the night period, declining thereafter to day-time levels. Furthermore, although the experimental procedure for both species was identical, *Astacus* has a single period of glucose increase, whereas the hyperglycemic period of *Orconectes* comprises two successive peaks. We are still puzzled about the physiological significance of the latter phenomenon.

The circadian rhythm of the blood CHH levels shows a pattern similar to that found for the blood glucose: basal

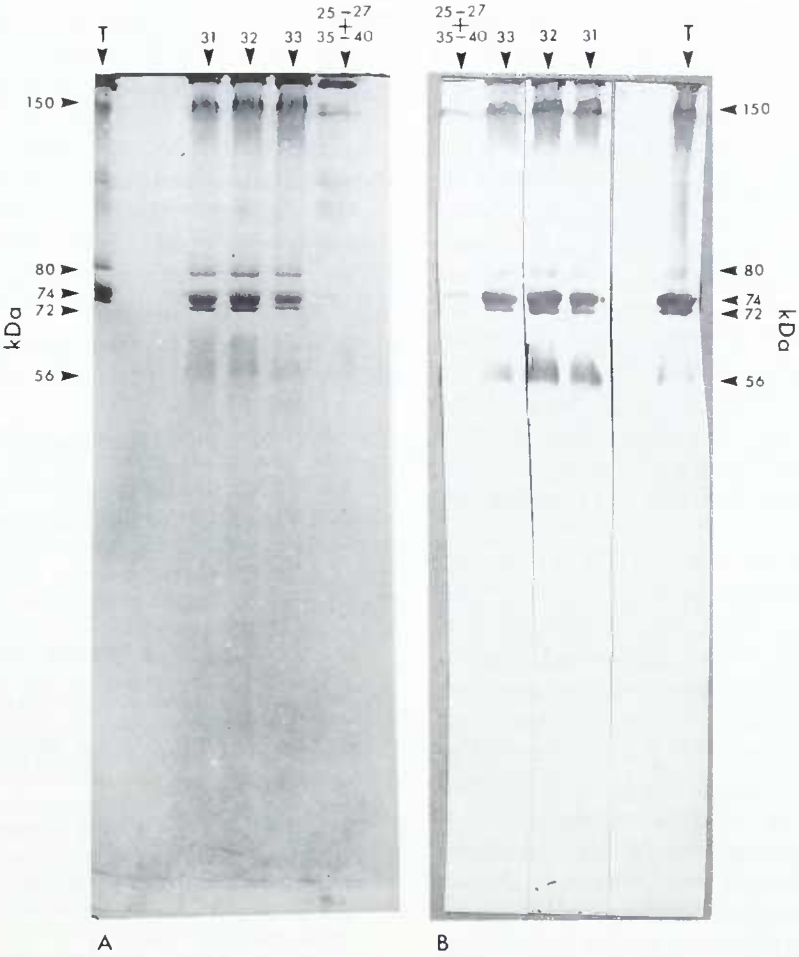

Figure 3. Results obtained after SDS-PAGE followed by immunoblotting of crude hemolymph and hemolymph gel filtration fractions from the crayfish (*Orconectes limosus*). T = total hemolymph; 31, 32, 33 = immuno- and bioactive hemolymph fractions; 25–27 and 35–40 = immuno- and bioactively negative hemolymph fractions. (A) Coomassie Brilliant Blue staining. (B) Immunoblotting with anti-*Orconectes*-CHH mouse serum.

levels during daytime and an increase of blood CHH content during the first hours of darkness. In previous studies on the diurnal cycle of the CHH cells in *Astacus*, we gathered information on the secretory dynamics of the perikarya and the rate of exocytoses of CHH granules, both events preceding nocturnal hyperglycemia (Gorgels-Kallen and Voorter, 1985). The increased blood CHH content described in this paper for *Orconectes* occurs in the period of expected high exocytosis of CHH into the hemolymph.

Our results show further that the application of a DAS-ELISA is a suitable method for determining hormone levels in crustacean hemolymph. Previously, the ELISA-technique was successfully applied by Quackenbush and Fingerman (1985) to determine the level of black pigment dispersing hormone (BPDH) in the blood of the fiddler crab. Our ELISA results are presented as optical densities. If we compare those with a standard curve of purified CHH, blood CHH levels during the day are estimated at about 1 ng per 100 μl hemolymph. The nocturnal peak in blood CHH content is comparable to about 10 ng purified hormone per 100 μl hemolymph. However, superfusion experiments have shown that the released bioactive CHH-peptide undergoes molecular changes that increase its potency relative to the storage pool in the sinus gland (unpub. obs.). Furthermore, our results point to the affinity of the released CHH to high molecular weight proteins in the hemolymph. We can only speculate about the possible effect of these molecular changes on the immunodetectability of the hormone in the blood. Therefore we cannot presently draw any conclusions about the actual quantity of bioactive hormone in the hemolymph.

Previous research on the chemical nature of CHH has focussed on the isolation, characterization, and physiological effects of CHH material in the sinus gland. In this neurohemal organ, the agent causing hyperglycemia in various species of decapod crustaceans has been described primarily as a neuropeptide with a molecular weight of around 7000 Da (for a review see Kleinholz, 1985). Kegel *et al.* (1989) described the amino acid sequence of the CHH (8524 Da) from the crab *Carcinus maenas.* Recent work in our laboratory on the sinus gland of the lobster *Homarus americanus* has shown that several CHH and CHH-like molecular forms occur in this organ (Tensen *et al.*, 1989). Furthermore, limited research on the chemical nature of newly synthesized CHH points to the presence of a prohormone or precursor in the perikarya (Stuenkel, 1983; Van Wormhoudt *et al.*, 1984a, b; Kallen *et al.*, 1986). Weidemann *et al.* (1989) sequenced the cDNA encoding a precursor for the CHH from the crab *Carcinus maenas.*

Although we know much about CHH in the sinus gland, our knowledge of the chemical nature of the CHH material released into the hemolymph is extremely limited. Our results have frequently pointed to substances of high molecular weight in the hemolymph that show strong affinity for hyperglycemic factors from the sinus gland. For instance, purification of sinus gland extract by gel filtration has always resulted not only in the purification of a hyperglycemic factor with a molecular weight of around 6500 Da, but also in immunological and biological activity in the void volume. Moreover, mixing the purified 6500-Da material with hemolymph always caused the low molecular weight form to disappear, leaving the immuno- and bioactivity exclusively in the high molecular weight void volume fraction (unpub. obs.). These observations, together with the strong immunopositive reaction in the hemolymph, encouraged us to search for more information about the molecular characteristics of the circulating hyperglycemia-producing material in the blood.

Our preliminary results presented in this paper show that, after gel filtration of hemolymph on Sephadex G-200 sf, high molecular weight proteins are detectable with both a CHH-immunopositive reaction and a strong hyperglycemic activity. Moreover, the bioactivity of released CHH is far higher than that of the CHH stored in the sinus gland. This might be caused by molecular changes in the hyperglycemic hormone just before or after release. Stuenkel and Cooke (1988) suggested that only small amounts of neurohormones must be released into the blood to meet the physiological needs. These authors suggest the presence of a "readily releasable pool" that is distinguishable from the bulk of stored material. Our results are consistent with this idea. They also point to the existence of a large non-active storage pool, as opposed to a small amount of bioactive neurohormone in the neurohemal organ. The high bioactivity of the CHH in the blood could also be caused by binding of the released factor to a carrier-protein. The results of SDS-PAGE and immunoblotting show immunoreactivity associated with several proteins of high molecular weight: 150, 80, 74, 72, and 56 kDa. The pattern of this electrophoresis corresponds to the electrophoretic behavior of crustacean hemocyanins as described by Markl *et al.* (1979).

Our standard analytical methods did not reveal any low molecular weight CHH-active proteins, but the possibility of their presence should not be excluded. We intend to continue the search for the role of subunits of hemocyanin as potential carrier-proteins for bioactive CHH.

Acknowledgments

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Biochemical and Functional Effects of Sulfate Restriction in the Marine Sponge, *Microciona prolifera*

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Abstract. The functional and biochemical consequences of sulfate restriction were studied in chemically dissociated *Microciona* sponge cells maintained in artificial seawater with or without SO_4^{2-}. In cells pre-treated to reduce pre-formed secretions, SO_4^{2-} deprivation reduced cell motility judged by the lack of aggregates in rotating or stationary cultures in comparison with controls. Microscopic examination showed that cells that customarily demonstrate cytoplasmic processes, such as filopodia and pseudopodia, exhibited marked decreases in these cellular processes when maintained in SO_4^{2-}-deprived artificial seawater. Uptake and incorporation of $^{35}SO_4^{2-}$ by disaggregated and pre-treated cells was higher under SO_4^{2-}-free conditions relative to controls; this effect was time dependent, rising to a maximum at 12 h, when a three- to seven-fold difference could be demonstrated. ^{3}H-leucine incorporation indicated that protein synthesis was similar in test and control populations. Comparative high voltage electrophoresis of supernatants containing $^{35}SO_4$ macromolecules from chemically dissociated cells indicated deficiencies of such $^{35}SO_4$ macromolecules if the rotated cells that released these secretions had been pre-treated in SO_4^{2-}-free artificial seawater.

The results of SO_4^{2-} restriction suggest that secretion of macromolecules or *Microciona* aggregation factor (MAF), and aggregation and locomotion of *Microciona* cells depend upon an adequate extracellular source of SO_4^{2-}, sulfate transport, and sulfation of macromolecules such as polysaccharides.

Introduction

Both vertebrate and in vertebrate cells require sulfated macromolecules on cell surface receptors and in intracellular fluid (Cassaro and Dietrich, 1977; Hogsett and Quantrano, 1978; Mulder, 1981; Klebe *et al.*, 1986; Mulder *et al.*, 1987). For example, mesenchymal migration of sea urchin embryos is blocked *in situ* in sulfate-deprived medium (Katow and Solursh, 1981), and cell motility and morphology in cell cultures have been influenced by sulfated glycosaminoglycans (Venkatasubramanian and Solursh, 1984). Blebbing has been observed on cell surfaces of sea urchin embryos maintained in sulfate-free seawater, but not the prolonged processes that accompany mesenchymal cell migration. In this instance it appeared that sulfate deprivation was capable of causing an inhibition of the formation of stable cell attachments to the basal lamina (Venkatasubramanian and Solursh, 1984; Akasaka *et al.*, 1980). The defect could be reversed by a 6-h pre-treatment in normal seawater.

Sulfate availability appears to be particularly important during early embryogenesis and differentiation in several species (Cassaro and Dietrich, 1977; Katow and Solursh, 1981; Lindahl, 1942; Immers and Runnstrom, 1965; Wenzl and Sumper, 1981). Particularly vital in this regard are the sulfated mucopolysaccharides; their presence correlates well with tissue-level organization and normal development (Wenzl and Sumper, 1981; Kinoshita and Saiga, 1979; Yamaguchi and Kinoshita, 1985).

This interesting background prompted us to address sulfation, using as a model *Microciona*, a relatively well studied marine sponge (Humphreys, 1963, 1967; Henkart *et al.*, 1973; Burger *et al.*, 1975; Jumblatt *et al.*, 1980; Misevic and Burger, 1986; Misevic *et al.*, 1987). These sponges are multicellular, but the relatively loose organization of embryonic and differentiated cells is easily disaggregated. If divalent cations are deleted from the

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supporting medium (seawater), a specific aggregation factor (AF)—a sulfated proteoglycan-like molecule—is released; this factor can then promote specific cell aggregation (Humphreys, 1963). AF contains two functional domains, one a cell binding portion, the other an AF interaction domain (Misevic and Burger, 1986). Cell aggregation by *Microciona* AF (MAF) appears to be based on multiple low affinity carbohydrate-carbohydrate interactions (Misevic *et al.*, 1987). The role of sulfate in such reactions is unknown, but other work shows that the migration and release from cells of proteoglycan-containing vesicles may be related to a high sulfate content (Albedi *et al.*, 1989; Takagi *et al.*, 1989). Monoclonal antibodies raised against sulfated proteoglycan from rat chondrocytes were used with immunoperoxidase electron microscopy to demonstrate relatively high concentrations of membrane-associated sulfated proteoglycans in cell processes and filaments from which matrix vesicles are presumably released into the surrounding medium (Takagi *et al.*, 1989).

In the studies to be described, the effects of seawater, with and without sulfate, upon subsequent aggregation of disaggregated *Microciona* sponge cells was examined, both in rotating and stationary cultures. The isolation of cells in a relatively simple culture medium, such as seawater, possesses advantages over such alternatives as perfusion, or *in vivo* methods, or the use of complex culture media. The level of sulfation can be controlled, all the relative enzyme systems are present in the cells, and the cellular uptake of labeled sulfur can be studied. Our primary purpose in the initial study was to observe the effects of sulfate deficiency, or restriction, upon cell process formation and locomotion and upon cellular aggregation. Using the conditions indicated by these observations, the incorporation of $^{35}SO_4$ was carried out, and correlations with intracellular-free sulfate and active sulfate (PAPS, *i.e.*, phosphoadenosine-5′-phosphosulfate) and sulfated macromolecules determined.

Materials and Methods

Sponges

Live specimens of *Microciona prolifera* were collected by members of the Supply Department of the Marine Biological Laboratory (Woods Hole, Massachusetts) during the months of July and August. Sponges were used on the day of collection or on the following day, but could be maintained in satisfactory condition for several days in the laboratory at ambient temperature in tanks of running seawater.

Buffers and artificial seawater preparations

Bicarbonate buffered artificial seawater (MBLSW) was made up according to the Marine Biological Laboratory formula (Humphreys, 1963; Cavanaugh, 1964). Calcium- and magnesium-free seawater (CMFSW) was prepared as described by Humphreys (1963). In aggregation assays, CMFSW was supplemented with 10 m*M* $CaCl_2$. Sulfate-free seawater was prepared as follows: in the case of MBLSW, magnesium chloride was substituted for $MgSO_4 - 7H_2O$; in CMFSW, sodium chloride was substituted for Na_2SO_4. The preparations were termed MBL − SO_4 and CMF − SO_4, respectively.

Dissociation of sponge cells

The chemical dissociation of *Microciona* cells (Humphreys, 1963) began with small lumps of tissue that were first rinsed to remove foreign material, and then blotted. Fragments (1–3 mm) were cut and placed in cold CMFSW in the ratio of 1 g/100 ml CMFSW. We dissociated the fragments by pressing them gently through no. 25 bolting cloth into a second volume of CMFSW. The resulting suspension contained about 2×10^7 cells/ml as estimated by hemocytometer counts. The suspension was spun in the centrifuge for 5 min at 2000 RPM and resuspended to make a concentration of 10^7 cells/ml. Small clumps were flushed gently with a Pasteur pipet and thereby readily broken up. The suspension was then rotated in CMFSW at 16°C for 6 h. The supernatant containing aggregation factor (AF) was removed, and the cells were washed and resuspended. The rotation was then repeated, first for 6 h in CMF − SO_4 and then for 24 h in MBL − SO_4. The cells were then divided into two aliquots: one was maintained in MBL − SO_4 for an additional 24 h, and the other was placed simultaneously in MBLSW for 24 h. The preconditioned cells from both aliquots were then pelleted and each aliquot resuspended in MBL − SO_4 and used in isotope labeling experiments.

Aggregation factor

AF was extracted and purified according to Humphreys (1963), as modified by Jumblatt *et al.*, (1980). Protein was estimated using the Bio-Rad colorimetric assay (Bradford, 1976).

Sponge cell aggregation assays (Humphreys, 1963; Jumblatt et al., *1980)*

Serial two-fold dilutions of AF were incubated for 20 min at 22°C with cells (10^7/ml) in the presence of $CaCl_2$. The cells were then visually inspected for evidence of aggregation.

Sponge cells in rotation-and petri dish cultures used to study cell motility and aggregation

Cells were prepared as described above, and batches were then adjusted to a concentration of 10^7/ml in the following buffered solutions: MBLSW, MBL − SO_4, and

CMFSW, CMF − SO_4. From each suspension, one aliquot was rotated in covered beakers for 24 h at 16°C. A second aliquot from each suspension was placed in glass petri dishes and maintained motionless at 22°C for 24 h. The presence of aggregates was then determined.

Microscopic studies

Following the incubation period, small aliquots of cultured preparations were mounted on glass slides, and overlaid with cover slips, which were sealed with resin to prevent evaporation. Some cell preparations were vitally stained with a 0.1% aqueous solution of Nile blue sulfate (Leith and Steinberg, 1972). The cells were examined by phase contrast and interference contrast microscopy with a Zeiss Axiophot microscope at magnifications of 100× and 400×.

Sulfate incorporation studies using isotope-labeled, carrier-free sulfuric acid ($H_2{}^{35}SO_4$)

Radiolabeled $H_2{}^{35}SO_4$ (2 mCi/ml) was purchased from New England Nuclear. Aliquots of each preconditioned *Microciona* cell preparation in MBLSW and MBL − SO_4 were washed in MBL − SO_4, then calibrated to 10^7 cells/ml in sulfate-deficient artificial seawater, and incubated in rotating culture in medium containing 2 μCi/ml $H_2{}^{35}SO_4$. Replicate 1-ml aliquots of cells were placed on 25-mm diameter cellulose acetate filter discs (0.45 μm), beginning at 15 min, and at intervals thereafter up to 12 h. The dried discs were treated as follows: (a) for uptake studies, duplicate dried discs were each placed in a scintillation vial and 15 ml Aquosol-2 liquid scintillation fluid added; (b) for incorporation studies, filter discs were treated with 100% ethanol to precipitate proteins, washed twice in ethanol, dried, and treated as in (a). Counts were carried out in a Beckman LS6000 IC scintillation counter. The results are expressed as dpm/10^7 cells.

High voltage electrophoresis (HVE)

Channels (2″ wide) were pencilled on Whatman 3M filter paper (18 × 22″), which was then moistened with 1% sodium tetraborate pH 9.1. One aliquot could then be spotted on one channel for a total of nine assays on each sheet of moistened paper. Electrophoresis was carried out at 1 kV and 180 mAmp for 60 min; the current was then discontinued and the paper dried in a warm air oven. Each channel, containing one separated extract, was cut into one-inch strips and placed in vials to which was added Beckman Redi-Solv EP scintillation fluid for scintillation counting as described.

$^{35}SO_4$ Incorporation into secreted extracellular macromolecules

Microciona cell suspensions were pre-treated and chemically dissociated, as described. The suspension medium was either CMFSW or CMF − SO_4. To suspensions adjusted to a concentration of 10^7 cells/ml in a volume of 50 ml, was added 100 μCi of carrier-free $^{35}SO_4$; the suspensions were rotated for 12 h at 16°C. The supernatants were harvested, and concentrates were prepared and assayed for MAF (16). The MAF pellets were washed exhaustively, redissolved in a minimal volume of artificial seawater, adjusted to equal protein concentrations, and dialyzed overnight in electrophoresis buffer. Such preparations were assayed by HVE. Free $^{35}SO_4$ and PAP (^{35}S) were included in the assays as reference standards. Material that remained at the origin following electrophoresis was regarded as containing macromolecules that had incorporated $^{35}SO_4$. Results are expressed as dpm/mg protein.

Amino-acid incorporation using ^3H-leucine

Aliquots of cell preparations maintained in the presence or absence of sulfate were calibrated to 10^7 cells/ml and incubated in MBL − SO_4 in the presence of 100 μl of a 50 μCi/ml solution of ^{3}H-leucine (>300 mCi/mmol—New England Nuclear); aliquots were taken for counts at spaced times beginning at 2 min. The cells were treated with 100% ethanol as described above.

Results

Sponge cell aggregation

In contrast to cells suspended in sulfate-containing medium, aggregation was either partially or greatly retarded in samples suspended in sulfate-free seawater. This could be demonstrated as follows: (1) test cells that were prepared in the routine manner were rotated at 16°C in the presence of $CaCl_2$ along with supernatants derived from an equal number of chemically dissociated cells rotated in either CMFSW or CMF − SO_4. Supernatants prepared in sulfate free seawater (CMF − SO_4) proved relatively ineffective in aggregation assays when compared with the action of AF or supernatant derived from sponge cells that had been rotated in CMFSW (Fig. 1).

(2) AF prepared from fresh cells under normal conditions and concentrated, was tested in routine assays with chemically dissociated cells rotated in either CMFSW or CMF − SO_4. When cells which had been pre-treated in CMF − SO_4 were used in assay, the aggregation of sponge cells was reduced in comparison with cells that had been pre-treated in sulfate containing seawater (Fig. 2). A small rim of adherent cells that ordinarily collected at the liquid-air interface of the container was not observed in assays that included CMF − SO_4 treated cells.

Petri dish cultures

Chemically dissociated sponge cells were rotated in changes of CMFSW and washed with CMF − SO_4. Ali-

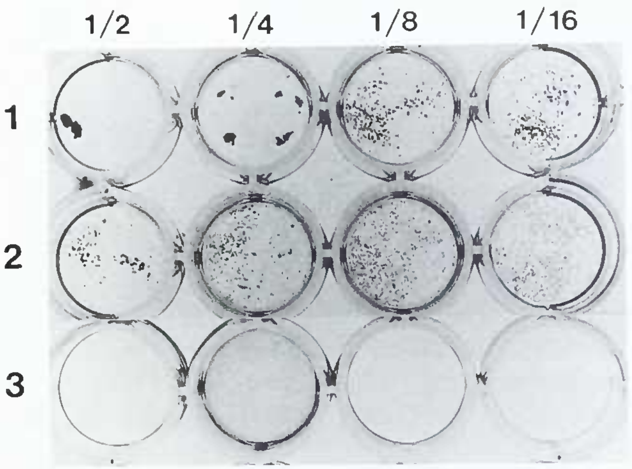

Figure 1. Results of aggregation assays using supernatants derived from chemically disaggregated cells rotated in sulfate-free artificial seawater. Fresh normally processed *Microciona* cells and $CaCl_2$ added. Photograph depicts results at 1 h. Wells from left to right in first two rows (1 and 2) depict serial dilutions of supernatant preparations. *Microciona* cells and $CaCl_2$ in CMFSW are in bottom-most (3) well (2nd from left). Reading from the top: Row 1, assay contains supernatant (MAF) from CMFSW cells; Row 2, contains supernatant from CMF − SO_4 cells. Aggregation is impaired in presence of CMF − SO_4 supernatant and dilutions.

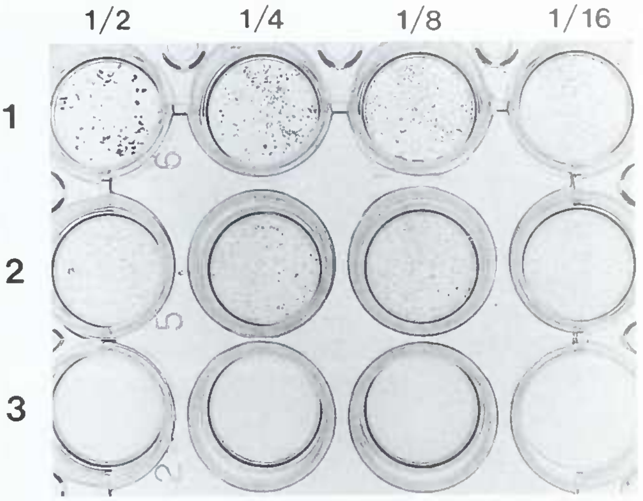

Figure 2. Aggregation assays using *Microciona* cells prepared from suspensions rotated in CMFSW or CMF − SO_4. Assays were carried out in calcified dilutions of MAF prepared as described (12, 16). Photograph depicts results at 1 h. Wells from left to right in rows 1 and 2 depict serial dilutions of MAF in CMFSW with $CaCl_2$. Wells at bottom contain samples of each cell preparation in calcified CMFSW. Reading from the top from left to right: first row, assay contains cells pre-treated in CMFSW; second row, cells pre-treated in CMF − SO_4. Aggregation is impaired in cells which had been pre-treated in CMF − SO_4.

quots of cells were then placed in even suspension in the following media: CMFSW, CMF − SO_4, MBLSW, MBL − SO_4. Each suspension was gently pipetted with a Pasteur pipet and counted; the count in each suspension was adjusted to 10^7/ml. Thirty ml of suspension were then poured into large glass petri dishes and gently pipetted to assure an even distribution of cells. The dishes were then covered and were permitted to remain undisturbed for 24 h at 22°C. Inspection at the end of this period revealed aggregates of varying sizes in samples that had been suspended in MBLSW and CMFSW. Aggregation was not observed in cell suspensions lacking sulfate (Fig. 3).

Microscopic studies

The most striking observation was a relative lack of cellular blebbing or of filopodia or pseudopodia in cell preparations suspended in sulfate-free seawater. In contrast, we commonly observed, in suspensions containing sulfate, slender filopodia that sometimes extended a long distance from single cells or from aggregates (Fig. 4A–D). Other, more substantial processes were suggestive of a cell elongating in the direction of a second cell using a single pseudopod as a means of locomotion. We observed occasional single cells, or cell clumps, from which multiple filopodia emerged, creating a stellate or radiating pattern (Fig. 4B). All of these appeared to be natural events in the presence of sulfate. They were greatly reduced or lacking in single cells and aggregates when sulfate was absent, whether Ca^{2+} and Mg^{2+} were present (Fig. 4E–F). Our general impression was that processes were most often seen in relatively agranular cells of intermediate to large size.

Incorporation of $^{35}SO_4$

The results of $^{35}SO_4$ uptake into *Microciona* cells are shown in Figure 5. Each sampling time point depicts the average of duplicate tests, and the values are expressed as

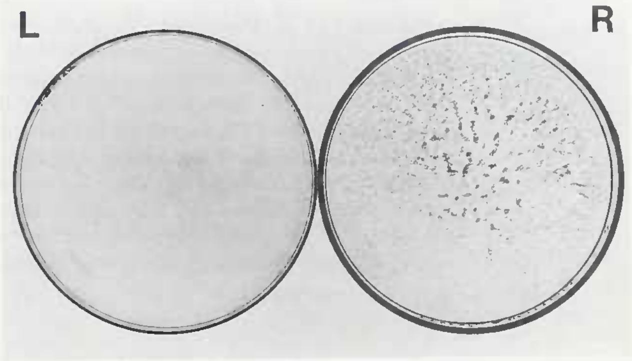

Figure 3. Aggregation of *Microciona* cells maintained in stationary culture in petri dishes. Readings are at 24 h under conditions as described in the text. Aggregation is observed when cells are maintained in MBLSW (right) but not when cells are in MBL − SO_4 (left).

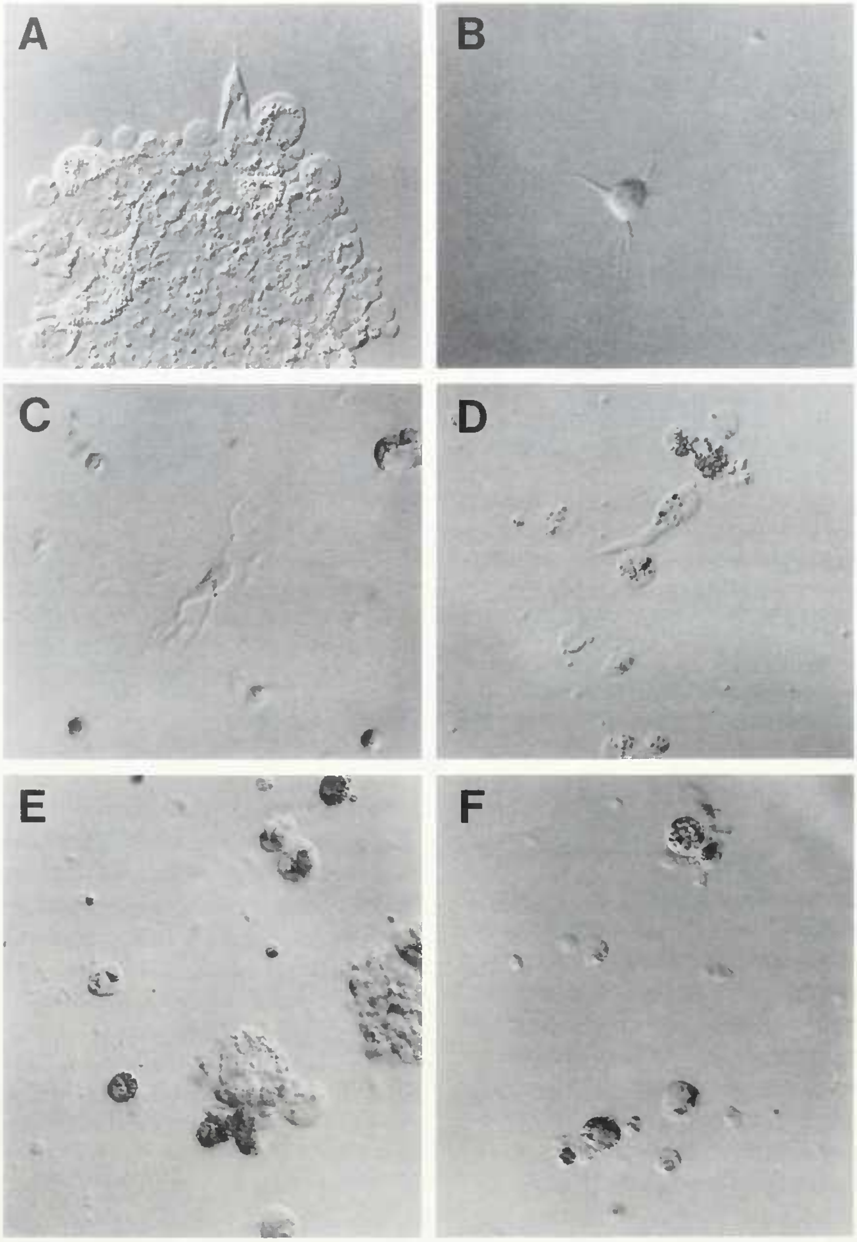

Figure 4. (A–B) *Microciona* cells observed by interference contrast microscopy 400× magnification. Cells shown here had been in rotation in MBLSW as described in text. Filopodia or pseudopodia, some of considerable length, are noted in cells in aggregates as well as single cells. Multiple filopodia produce a stellate appearance in cell or cells seen in Figure 4B. (C–D) Interference contrast study of *Microciona* cells 400× magnification. Cells shown here had been maintained stationary in petri dish cultures in MBLSW as noted in text. Filopodia or pseudopodia are frequent in single cells and in small aggregates. (E) Interference contrast study of *Microciona* cells which had been in rotation in MBL − SO_4 as described in text. Typically, small aggregates were produced in the relative absence of filopodia or pseudopodia. (F) Interference contrast microscopy of *Microciona* cells which had been maintained stationary in MBL − SO_4 in petri dishes as described in text. The majority of cells were unattached, lacking processes, or adherent in very small clusters.

dpm per 10^7 cells. The highest uptake of $^{35}SO_4$ occurred in cell samples pre-treated in sulfate-free medium; in comparison, cells pre-treated in sulfate-containing medium demonstrated considerably lower levels of $^{35}SO_4$ uptake beginning minutes after the addition of $^{35}SO_4$. In replicate experiments with sponge derived from two collecting stations, cells in MBL − SO_4 showed progressive increases in $^{35}SO_4$ uptake and incorporation up to 12 h,

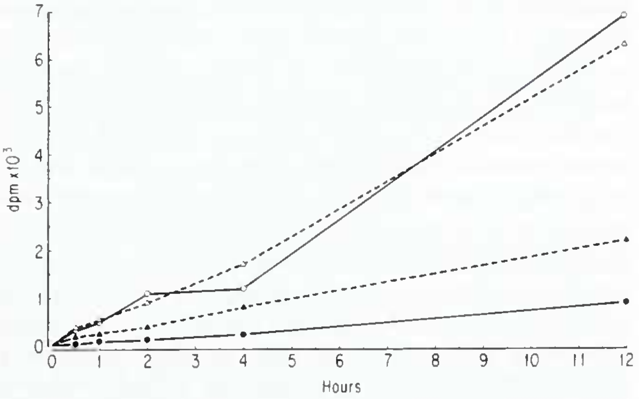

Figure 5. Uptake of $^{35}SO_4$ by *Microciona* cells. Pre-treated *Microciona* cells were distributed into flasks containing (a) MBLSW, (b) MBL − SO_4 at a concentration of 10^7 cells/ml and placed in rotation for 24 h. Centrifuged pellets from each flask were washed with MBL − SO_4 and suspended in MBL − SO_4; 5 μl $H_2{}^{35}SO_4$ (carrier free) was added to each flask and rotated at 16°C. Radioactivity of duplicate 1-ml aliquots was monitored at intervals up to 12 h. The solid lines depict uptake of $^{35}SO_4$ by cells pre-treated in MBL − SO_4. The broken lines represent cells pre-treated in MBLSW. Circles and triangles signify experiments carried out on cells from sponge obtained at two different collecting stations.

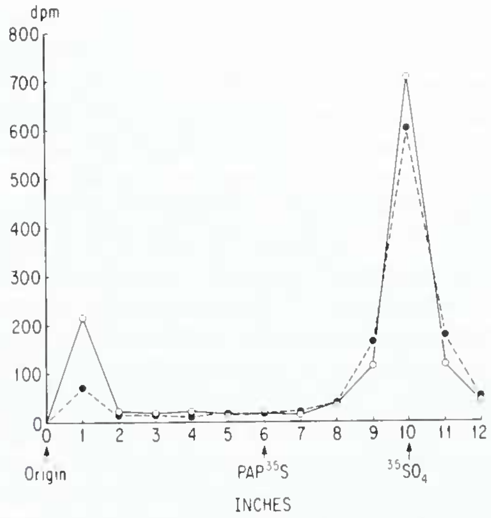

Figure 6. High voltage electrophoresis of $^{35}SO_4$ macromolecules in supernatant preparations derived from rotated chemically dissociated *Microciona* cells with incorporated carrier free $^{35}SO_4$. 75 μl placed at origin. Conditions: 1% sodium tetraborate pH 9.1, 1 KV, 180 mA, 1 h. CMFSW □---□; CMF − SO_4 ■---■

in contrast to controls. At 12 h, values for $^{35}SO_4$ were three- to eight-fold higher in sulfate deprived cells than in controls (6925 and 6603 dpm in test samples versus 882 and 2075 dpm in controls). Incorporation of $^{35}SO_4$ into macromolecules was calculated to be 70–85% of $^{35}SO_4$ uptake at the 12-h sampling time. In recent separate experiments with extended sampling times, the differential in counts between cells pre-treated with MBLSW and with MBL − SO_4 remained for up to 5 days, at which time sampling was discontinued. In samples of cells pre-treated with MBL − SO_4, elevated counts exhibited some fluctuations, but remained at high levels during the period of sampling. Cells were collected for extracts that will be analyzed by HVE for the distribution of $^{35}SO_4$ macromolecules. This study will be reported in a separate publication.

$^{35}SO_4$ Incorporation into secreted extracellular macromolecules

The protein yields in the extracellular secretion from 5 × 10^8 *Microciona* cells were as follows: from CMFSW cells, 1.12 mg; and from CMF − SO_4 cells, 0.37 mg. The yield of $^{35}SO_4$ macromolecules revealed by HVE after subtraction of background values was: for CMFSWMAF, 190 dpm/50 μg protein; for CMF − SO_4 supernatant, 35 dpm/50 μg protein (Fig. 6).

Incorporation of ^3H-leucine

Incorporation of ^3H-leucine into protein by *Microciona* cells in sulfate-free seawater was comparable to that of cells rotated in sulfate-containing seawater; aliquots from specimens obtained at two collecting stations were examined. In all instances, incorporation was prompt, with mild to moderate increases over the testing period (Table 1).

Discussion

A role for sulfated polysaccharide recognition in sponge cell aggregation was suggested by Coombe *et al.* (1987) based upon an analysis of endogenous polysaccharide

Table 1

^3H-leucine incorporation into Microciona *cells maintained under different conditions of sulfate availability*

Conditions of culture	Time of sampling	^3H-Leucine incorporated (dpm)
1a. MBL + SO_4	30 min	17111*
	2 h	20586
1b. MBL − SO_4	30 min	19149
	2 h	25644
2a. MBL + SO_4	30 min	13688
	2 h	21646
2b. MBL − SO_4	30 min	12140
	2 h	19781

* Average of duplicate ethanol precipitated samples after subtraction of background values.

10^7 cells per sample.

Specimens 1 and 2 were obtained at two different collecting stations.

from sponge cell cholate lysates. The extract possessed a high content of sulfate and inhibited the aggregation of intact sponge cells, as did the sulfated compounds polyvinyl sulfate and dextran sulfate. The latter compounds, coupled to erythrocytes, rendered the erythrocytes agglutinable in the presence of sponge cell lysates.

In the present studies, pre-treated chemically dissociated cells maintained in a sulfate-free environment exhibited greatly reduced motility and marked changes in functional behavior. Aggregation of *Microciona* cells became impaired under these conditions, particularly in stationary cultures, regardless of the presence of Ca^{2+} and Mg^{2+}. The effect of a sulfate-free environment could be observed in *Microciona* cells, as well as in supernatants derived from cells under sulfate-free conditions. This was demonstrated in controlled aggregation assays, as follows. (1) The capability of AF derived from cells in CMFSW was compared with that of supernatants derived from cells chemically dissociated in $CMF - SO_4$ with healthy *Microciona* cells and $CaCl_2$ being employed in the assays; and (2) cells prepared in sulfate-free artificial seawater were tested in standard assays with AF prepared in the usual manner from chemically dissociated cells. Of special note was the finding that aggregates were absent or greatly reduced in size when cells under these conditions were maintained in stationary cultures. Random collision in rotation cultures probably accounted for the small aggregations noted, but even in this circumstance, aggregation in the absence of sulfate was modest when compared with *Microciona* cells maintained in sulfate.

The most obvious morphologic change in individual cells maintained in sulfate-free medium was a reduction in cell processes such as filopodia and pseudopodia. The relative lack of such processes most likely contributed to the inability of these cells to form normal contacts, especially when in stationary cultures. In the presence of sulfate, the primary activity seemed to reside in medium- to large-sized cells, cells which were relatively agranular, some of which were reminiscent of choanocytes (Kuhns *et al.*, 1980). It remains to be established whether certain cell types in *Microciona*, such as larval cells, possess special components that are unusually sensitive to sulfate deprivation, and that are necessary for secretion or cellular migration, as is the case in the sea urchin embryo.

The secretion of sulfated polysaccharide appears to be necessary to maintain these functions, as established earlier by Immers and Runnstrom (1965), and this form of secretion is diminished or sulfate-poor in parallel with sulfate restriction. However, there is no direct evidence from our work that sulfated polysaccharides were specifically affected by changes in seawater sulfate content. The evidence presented relates to the flow of sulfate into cells, and the biosynthesis of sulfated macromolecules as important factors in cell locomotion and cell adhesion for reasons that are yet unclear. Nevertheless, we presume that MAF molecules become sulfated as part of the biosynthetic process (Misevic *et al.*, 1987). Although the design of this study does not enable conclusions about their specific nature, $^{35}SO_4$ macromolecules appeared to be deficient in *Microciona* supernatants derived from chemically disaggregated cells, in contrast to supernatants derived from cells in sulfated seawater. When supernatants derived from sulfate-free cells were concentrated and purified as described, the protein content, as well as the content of macromolecules (defined on HVE), were reduced in relation to AF prepared from an equal number of *Microciona* cells prepared in artificial seawater. Such a deficit may be caused by a defective transport of secretory vesicles to the cell surface in the absence of sulfate.

We suspect, from our results, that sulfation of macromolecules such as polysaccharides may be crucial in the trans-golgi transport of vesicles and their secretion into the extracellular matrix. These results, coupled with findings that sulfate depleted cells can greatly augment $^{35}SO_4$ incorporation relative to controls, suggest a mechanism whereby extracellular sulfate deficiency can alter the sulfation process, perhaps by influencing membrane composition and function. Note in this context that amino acid uptake and incorporation was similar in test and control cells as judged by experiments using ^{3}H-leucine.

Our studies of sulfate depleted cells suggest that the sulfate assimilatory pathway is altered when sulfate becomes rate limiting. Regulation and transport in such a system has been explained by the existence of a specific membrane permease in two bacterial species, *Salmonella typhimurium* and *Anacystis nidulans* (Green *et al.*, 1989); the permease genes have been cloned and, from these, a polypeptide structure of a putative membrane component determined. Homologies with message derived from sulfate-restricted *Microciona* sponge might be sought using probes derived from these bacteria. Studies in sulfate-deficient wheat and barley roots have also defined a sulfate transporter that was sensitive to DIDS, an inhibitor of anion transport (Clarkson and Saker, 1989). A sulfate permease, if defined in our system, would encourage further studies to define ways in which cells recognize sulfate levels and transduce this signal into altered sulfate transport, increased biosynthesis of sulfated glycoconjugates and altered cell locomotion. Fine structure differences in MAF derived from sulfate-deprived versus normal cells may prove important in defining extracellular prompting mechanisms which initiate or modulate such changes (Brunner, 1977).

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Extracellular Hemoglobins of Hydrothermal Vent Annelids: Structural and Functional Characteristics in Three Alvinellid Species

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Abstract. The polychaete annelids *Alvinella pompejana, Alvinella caudata,* and *Paralvinella grasslei* are strictly associated with deep sea hydrothermal vents. Each species possesses an extracellular hemoglobin, Hb, which has been studied and compared to that of a common intertidal polychaete, the lugworm *Arenicola marina.* The four Hbs exhibit very similar quaternary structures and spectral properties, and only small differences appeared in the gross polypeptide compositions after reduction and sodium dodecyl sulfate denaturation of the native molecules. Conversely, by a comparison of the effects of pH (6.6–7.6) and temperature (10–40°C) on their intrinsic O affinities, Bohr factors, cooperativities, and apparent heats of oxygenation, lugworm Hb can be differentiated from that of the alvinellids, and the Hb of *A. pompejana* from that of *A. caudata.* The known biology of the lugworm and a further analysis of the data suggest several hypotheses concerning the *in vivo* O_2 transport function of the alvinellid Hbs, the *in vivo* blood pH value in the two alvinellid species, their respective range of optimal temperature, and their ability to create a differentiated and stable external microenvironment.

Introduction

The known members of the tubicolous polychaete family Alvinellidae are associated only with deep sea hydrothermal vents. In the East Pacific Rise region, the tubes of the closely related species *Alvinella pompejana* and *Alvinella caudata* form honeycomb-like structures covering the external surface of the active vents, where they are frequently associated with the smaller species *Paralvinella grasslei* (Desbruyères and Laubier, 1986). The mixing of the very hot, anoxic vent water (up to 320°C) with the cold, oxygenated deep seawater (2°C) occurs at random. All three alvinellid species are supposed to live on the colder edge of a very sharp thermal gradient, at temperatures as high as 50°C (Desbruyères *et al.,* 1982; Arp and Childress *in* Terwilliger and Terwilliger, 1984). This environment is characterized by high-frequency, unpredictable changes in temperature, pH, oxygen partial pressure, and sulfide concentration (Johnson *et al.,* 1986, 1988).

The alvinellids have well-developed gills (Jouin and Gaill, 1990) and a closed vascular system containing a high molecular weight, extracellular hemoglobin (Hb) dissolved in the blood. These Hbs have rarely been studied, and most of the available data have been obtained by Terwilliger and Terwilliger (1984) on *A. pompejana* Hb. Recently, one of us (A.T.) collected fresh blood directly from living specimens of *A. pompejana, A. caudata,* and *P. grasslei.* We describe here the structure and some of the functional properties of the Hbs from these samples. The effects of pH and temperature on the oxygen binding properties of the Hbs were examined at constant inorganic ion concentration and at one atmosphere hydrostatic pressure. For comparison, the same studies were carried out on solutions of the extracellular Hb of a mainly intertidal species, the common lugworm *Arenicola marina,* prepared and stored in the same conditions.

Materials and Methods

Animals

The alvinellids were collected at 2600 m depth in November 1987 during the French-American "Hydronaut"

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expedition on the "13°N" hydrothermal vent site (East Pacific Rise region, Fustec *et al.*, 1987). Large pieces of black or white smokers were plucked off by the external arm of the DSRV *Nautile* and placed in an insulated, non-pressurized container, closed at depth to keep temperature constant as the yellow submarine surfaced. The lugworms were collected on the Penpoull beach near Roscoff, Brittany, France.

Blood collection and Hb solution preparation

Immediately after the alvinellids were recovered on board ship, they were opened dorsally, and the blood, uncontaminated with coelomic fluid, was withdrawn from the main vessels into glass micropipettes and pooled on melting ice. In Roscoff, the same procedure was applied to lugworms kept unfed for 12 to 24 h in local running seawater (temperature 14–16°C). The total blood volumes collected from the alvinellids were around 0.8 ml for *A. pompejana* (10 specimens), 0.7 ml for *A. caudata* (12), and 0.05 ml for *P. grasslei* (3).

The blood was centrifuged at low speed for a few minutes, and the supernatant was divided into two parts. (i) For examination of the Hb molecules by transmission electron microscopy (TEM), a few droplets of the supernatant were diluted 1:200 in a buffer comprising 50 m*M* Bis-tris-propane (BTP; Sigma) and HCl at pH 7.4. The grids were prepared by standard techniques (Valentine *et al.*, 1968), on board ship or in the Roscoff laboratory. (ii) The remaining supernatant was equilibrated against 50 m*M* BTP-seawater/HCl buffer (pH 7.6) by gel filtration on Sephadex G-25, saturated with carbon monoxide, and frozen in liquid nitrogen. In Paris, these samples were thawed, and a metHb-free, HbCO-free, pure HbO_2 solution was prepared using standard techniques (Riggs, 1981).

Spectrophotometric studies

U.V./vis. absorption spectra of the Hbs were obtained at 20°C with a Bausch and Lomb Spectronic 2000 spectrophotometer. The heme concentration of the solutions was determined using a millimolar extinction coefficient $\epsilon = 11.0$ at 540 nm for the cyanmet heme (Van Assendelft, 1970).

Electrophoretic studies

The Hbs were denaturated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence or absence of mercaptoethanol (ME). The Hbs and markers (Pharmacia) of low relative molecular mass (M_r) were first heated at 100°C for 5 min in a 2.5% SDS solution, with or without 5% ME. The electrophoresis was then carried out on 10% polyacrylamide slab gels, in 12.5 m*M* Tris/glycine buffer (pH 8.5), with 0.1% SDS.

Functional properties

For studies of the O_2-binding characteristics of the Hbs, aliquots of the pure HbO_2 solutions were equilibrated against 50 m*M* BTP/HCl buffer by gel filtration on Sephadex G-25 (final heme concentration: 40–70 μM). The buffers were adjusted in order to obtain constant pH values: 6.6, 6.9, 7.25, 7.6, whatever the experimental temperature: 10, 20, 30, 40°C. Except for Na^+, which varied between 265 and 305 m*M* depending mostly on the pH value, the inorganic ion concentrations, in m*M*, were also kept constant: $Cl^- = 470$; $SO_4^{2-} = 30$; $Mg^{2+} = 50$; $Ca^{2+} = 10$. These values are similar to those observed in the coelomic fluid of the lugworm (Robertson, 1949). The total osmolarity of the solutions was about 1.06 OsM.

Oxygen-equilibrium curves (OEC) were obtained, with no carbon dioxide in the gas phase and at one atmosphere hydrostatic pressure, by a continuous spectrophotometric method; we used a Hemox Analyser spectrophotometer (TCS, Southampton, Pennsylvania) interfaced with a Hewlett-Packard 85B microcomputer and a Hewlett-Packard ColorPro Graphics plotter. The purified HbO_2 solution was first equilibrated against pure oxygen and then slowly deoxygenated with pure nitrogen or argon. The deoxygenation procedure lasted 60 to 90 min, and the microcomputer was programmed to store up to 300 points of the OEC on tape, each point corresponding to the coupled mean values of 30 and 60 successive measurements of, respectively, oxygen partial pressure (P_{O_2}) and O_2 saturation of the Hb. Negligible quantities of metHb were produced during these experiments, a consequence of the particularly high resistance of lugworm and alvinellid Hbs to oxidation (Toulmond *et al.*, 1988). The P_{O_2} at half saturation of the Hb (P_{50}) was calculated from the experimental values between 40 and 60% O_2 saturation by linear regression analysis, and approximate values of the dissociation constants for the R and T states [respectively, K_R and K_T (Edelstein, 1975)] were estimated graphically from the Hill plot of the OEC. The value of the Hill coefficient [n_{max}, corresponding to the maximum slope of the Hill plot (Imai, 1982)], as well as its position on the saturation axis, were estimated graphically from the calculated first derivative of the Hill plot, the so-called cooperativity curve (Girard *et al.*, 1987).

The Hemox technique gave highly reproducible results, especially in conditions where the Hb affinity is high. The statistical analysis of a preliminary set of 10 OECs, obtained at pH = 7.6 and 20°C on lugworm blood, gave the following mean results (value ± SD): P_{50} (mm Hg) $= 1.82 \pm 0.04$; $n_{50} = 2.36 \pm 0.06$; K_T (mm Hg) $= 16.1 \pm 1.1$; K_R (mm Hg) $= 1.21 \pm 0.32$.

Results

Absorption spectra

Absorption spectra were typical of hemoglobins and quite similar in all the species studied; the position of

Table I

Spectral position in nm of HbO_2 α and β peaks and ratio of absorbance of the α to the β peaks in the four studied species

	Alvinella pompejana	*Alvinella caudata*	*Paralvinella grasslei*	*Arenicola marina*
Peak	574.5 0.2*	574.2 0.3	573.7	573.7 0.3
Peak	539.8 0.2	539.8 0.2	539.5	538.8 0.2
A_α/A_β	0.92 0.01	0.93 0.01	0.96	0.98 0.01
n**	8	8	2	8

* Standard deviation.

** Number of measurements.

major absorption peaks showed only slight, insignificant differences. Temperature and pH changes had practically no effect on these spectra. In all cases, even in the absence of methemoglobin, the ratio of absorbance of the α peak to the β peak was less than one (Table I).

Molecular structure

In the four species, the electron micrographs of negatively stained native molecules showed the same two-tiered hexagonal structure typical of annelid extracellular Hbs (Fig. 1); the dimensions were practically identical (Table II). FPLC filtration (Fig. 2) on a Superose 6 column (Pharmacia), as well as filtration on a Sepharose 6B column (1.6 × 70 cm), showed that alvinellid and lugworm Hbs have practically the same elution volume and, most probably, similar M_rs.

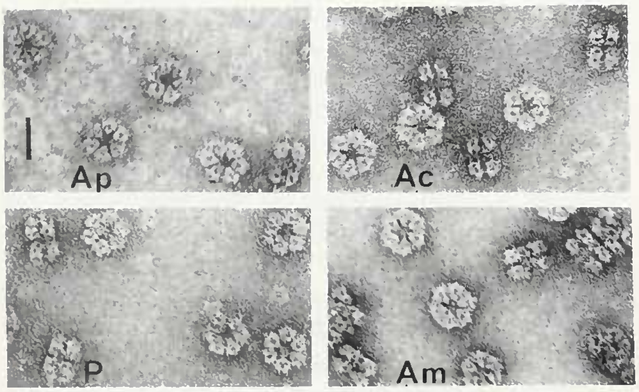

Figure 1. Electron micrographs of native molecules of the four extracellular Hbs, negatively stained with 2% uranyl acetate. Scale bar: 25 nm. (Ap) *Alvinella pompejana;* (Ac) *A. caudata;* (P) *Paralvinella grasslei;* (Am) *Arenicola marina*

Table II

Dimensions in nm of negatively stained Hb molecules as measured on electron micrographs

	Alvinella pompejana	*Alvinella caudata*	*Paralvinella grasslei*	*Arenicola marina*
Maximum diameter	30.4 0.8*	30.2 0.9	29.6 1.4	30.0 1.2
Side to side width	27.0 0.8	27.2 0.7	26.5 1.1	27.5 0.7
Height	19.7 0.9	19.4 1.2	18.9 1.9	19.7 1.0

* Standard deviation; n = 30.

In the three alvinellid species, denaturation and electrophoresis of the Hbs by SDS-PAGE yielded three major bands corresponding to proteins of M_r about 45,000, 30,000, and 15,000. Two fainter bands were also present corresponding to proteins of M_r *ca.* 28,000 and 22,000 in the genus *Alvinella,* and about 28,000 and 25,000 in the genus *Paralvinella.* By comparison, denaturation of *Arenicola marina* Hb gave four major bands corresponding to M_rs of about 45,000, 32,000, 28,000, and 15,000 (Fig. 3A).

In the four species, reduction by ME and simultaneous denaturation by SDS produced a major band corresponding to polypeptides of M_r between 14,000 and 16,000. Fainter bands corresponded to polypeptides of M_r about 35,000 and 25,000 in the genus *Alvinella,* 30,000 and 28,000 in *Arenicola,* and 28,000 in *Paralvinella* (Fig. 3B).

Oxygen equilibrium studies

Because so little *P. grasslei* blood was available, these studies were carried out only on *A. pompejana, A. caudata,*

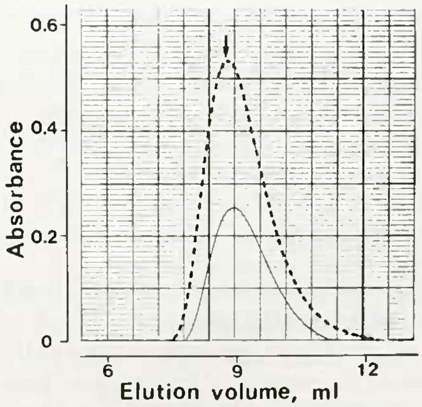

Figure 2. Elution profiles of *Alvinella pompejana* Hb on a Superose 6 column, in Bis-tris-propane/HCl buffer. The arrow indicates the peak position for *Arenicola marina* Hb. Absorbance was measured at 280 nm (solid line) and 410 nm (dashed line).

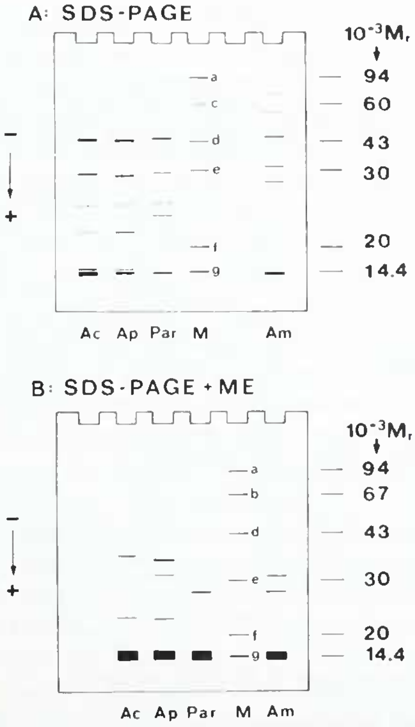

Figure 3. SDS slab gel electrophoresis, 10% polyacrylamide, of the four extracellular Hbs. (A) Before reduction by mercaptoethanol (ME); (B) after reduction by ME. (Ac) *Alvinella caudata;* (Ap) *A. pompejana;* (Par) *Paralvinella grasslei;* (Am) *Arenicola marina,* (M) low molecular mass markers (Pharmacia). (a) Phosphorylase; (b) serum albumin; (c) catalase; (d) ovalbumin; (e) carbonic anhydrase; (f) trypsin inhibitor; (g) lactalbumin.

and *Arenicola marina* Hbs. Figure 4 shows the Hill plot of a typical OEC obtained on *A. pompejana* Hb. *In vitro,* the alvinellid Hbs were characterized by a very high intrinsic O_2 affinity, with P_{50} values very dependent on pH and temperature (Table III). The normal Bohr effect was large, with Bohr factors that may have been lower than −1, and was greatest at low temperature and at low to medium O_2 saturation of the pigment (Table IV). The cooperativity was also high. The Hill coefficient, n_{max}, was in some cases higher than 4 (Fig. 5) and was strongly dependent on pH and temperature, being maximum for pH around 6.6–6.9 (Fig. 6). The apparent heat of oxygenation, ΔH, was also very high, peaking at more than −100 kJ/mol O_2, and strongly pH dependent (Table V).

The two alvinellid Hbs differed significantly with respect to these characteristics: the Bohr effect, cooperativity, and apparent heat of oxygenation were systematically higher in *A. pompejana* than in *A. caudata.* However their Hbs shared particular properties quite different from those of the lugworm. In the same experimental conditions, the lugworm Hb exhibited a lower O_2 affinity, a lesser Bohr effect with maximum values of the Bohr factor at medium to high O_2 saturation, a lower cooperativity with maximum values at rather alkaline pH (about 7.25–7.6), and lower pH-independent values of ΔH.

Discussion

Molecular structure

The alvinellid and lugworm Hbs exhibit the same quaternary structure, and it is typical of annelid extracellular Hbs. For the four molecules, and in the same experimental conditions: (i) FPLC, as well as low-pressure column chromatography, give almost identical elution profiles (Fig. 2) indicating very similar M_rs of about 3.6×10^6; and (ii) the native molecules measured on electron micrographs show only small, nonsignificant differences in dimensions (Table II). These M_rs and dimensions are very close to those recorded in the literature for the Hbs of intertidal polychaetes and terrestrial or aquatic oligochaetes (Vinogradov *et al.,* 1982), and they are very similar to those obtained in a recent small angle X-ray scattering study of lugworm Hb (El Idrissi Slitine *et al.,* 1990).

From these observations, we consider that the decrease in hydrostatic pressure experienced by the alvinellid blood during the submarine's rise to the surface (about 260 atmospheres) had little or no effect on the shape, size, structure, and, consequently, on the functional properties of alvinellid Hbs. Three observations support this opinion: (i) the electron micrographs show that both alvinellid and lugworm Hbs dissociate into more or less spheroidal particles, probably corresponding to twelfths of the native molecules; but the proportion of these particles is nearly

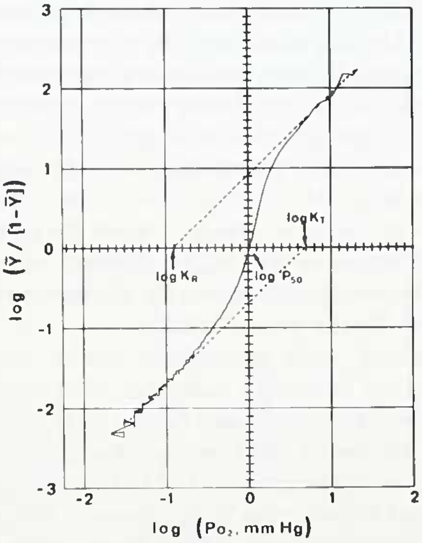

Figure 4. Hill plot of a typical oxygen equilibrium curve of *A. pompejana* extracellular Hb. Log K_R and log K_T were graphically estimated at the intersection of the log P_{O_2} axis by straight lines (slope = 1) drawn asymptotic to the Hill plot at extreme high and low O_2 saturation values, respectively. pH 6.90; 20°C; heme concentration: 70 μM

Table III

P_{50} in mm Hg as a function of pH, 6.6 to 7.6, and temperature, 10 to 40°C. Each value was obtained from one, rarely two, O_2-binding curves

		6.6	6.9	7.25	7.6
Alvinella pompejana	10°C	0.5	0.2	0.1	ND*
	20°C	1.9	1.0	0.3	0.2
	30°C	3.9	1.8	0.9	0.5
	40°C	8.1	4.4	2.9	2.2
Alvinella caudata	10°C	0.5	0.4	0.2	0.1
	20°C	1.8	1.0	0.4	0.3
	30°C	3.8	2.0	1.1	0.8
	40°C	6.6	4.4	2.5	2.4
Arenicola marina	10°C	5.7	4.2	2.8	1.4
	20°C	9.5	6.5	3.7	2.1
	30°C	13.9	9.1	5.4	3.3
	40°C	15.6	10.5	5.8	4.3

* Not done.

Table IV

Effect of temperature, 10 to 40°C, on the mean Bohr factor calculated between pH 6.6 and 7.6 for almost completely deoxygenated ($\phi T = \Delta \log K_T/\Delta pH$), half-oxygenated ($\phi P_{50} = \Delta \log P_{50}/\Delta pH$), and almost completely oxygenated ($\phi R = \Delta \log K_R/\Delta pH$) Hbs

Bohr coefficient		ϕT	ϕP_{50}	ϕR
Alvinella pompejana	10°C	−1.60	−1.17	−0.05
	20°C	−1.21	−1.18	−0.24
	30°C	−1.20	−0.89	+0.02
	40°C	−0.86	−0.56	−0.08
Alvinella caudata	10°C	−0.35	−0.76	−0.15
	20°C	−0.93	−0.90	−0.30
	30°C	−0.92	−0.68	−0.03
	40°C	−0.78	−0.47	−0.03
Arenicola marina	10°C	−0.18	−0.62	−0.44
	20°C	−0.34	−0.66	−0.43
	30°C	−0.34	−0.64	−0.38
	40°C	−0.44	−0.58	−0.38

the same for all species, indicating either their normal presence in the blood *in vivo*, or, most probably, their unavoidable formation during the preparation of the grids for the TEM study. (ii) A recent study has shown that hydrostatic pressure dissociates annelid extracellular Hbs significantly only when it is *increased* to more than 1000 atmospheres (Silva *et al.*, 1989); a *decrease* in hydrostatic pressure, from about 260 to 1 atmosphere, would be unlikely to substantially affect the quaternary structure of these Hbs. (iii) Preliminary experiments have shown that, during a progressive increase of the hydrostatic pressure up to 1500 atmospheres, followed by a progressive decrease back to one atmosphere, the absorbance spectrum of half-oxygenated lugworm Hb is not appreciably modified, indicating that no change occurs in either the O_2 saturation or the O_2 affinity of the Hb (Hui Bon Hoa and Toulmond, unpub.). Nevertheless, we must keep in mind that, on the basis of data obtained *in vitro* at 1 atmosphere hydrostatic pressure, we compare below the properties of Hbs that function *in vivo* at two different values of hydrostatic pressure (1 atmosphere for the lugworm Hb, 260 atmospheres for the alvinellid Hbs).

We obtained some information about the detailed structure of the native Hb molecules. SDS denaturation confirms that these molecules belong to the annelid extracellular Hb family, with only small variations around the general type (Vinogradov, 1980). However, small differences exist between the electrophoretic patterns of *A. pompejana* and *A. caudata* Hbs. These differences, together with those concerning the functional properties discussed below, confirm the distinct taxonomic status of these two recently separated species (Autem *et al.*, 1985; Desbruyères and Laubier, 1986).

Physicochemical and functional properties

Alvinellid Hbs can be easily distinguished from lugworm Hb in that there are notable differences of intrinsic O_2 affinity, Bohr effect, cooperativity, and apparent heat of oxygenation. In a detailed examination of these properties, the Hb of *A. pompejana* can be distinguished from that of *A. caudata*. Can these differences be correlated with what is known of the specific characteristics of the animals and their environment?

The high intrinsic O_2 affinity of *A. pompejana* Hb has already been reported by Terwilliger and Terwilliger (1984). We confirm here that the O_2 affinity of both *A. pompejana* and *A. caudata* Hbs is very high whatever the

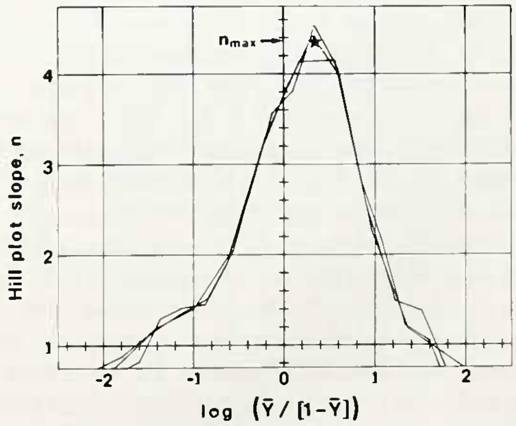

Figure 5. Three different calculations of the first derivative of the Hill plot of Figure 4, showing the variations of the Hill plot slope, *n*, as a function of log (Ȳ/(1 − Ȳ)). n_{max}: the graphically estimated value of the Hill coefficient.

experimental conditions (Table III), 2 to 10 times higher than that of the lugworm which has a Hb affinity for O_2 that is already quite high (for a comparison with other annelid Hbs, see Weber, 1980). In the extreme conditions of low temperature (10°C) and high pH (7.6), the O_2 affinity of *A. pompejana* Hb was so high (P_{50} lower than 0.1 mm Hg, with 1 mm Hg = 133.3 Pa) that it could not be measured with the Hemox technique. Hbs with high O_2 affinities are generally considered very adaptive in species lacking an efficient, specialized respiratory organ (see Weber, 1978). But alvinellid gills are characterized by the highest specific surface areas yet measured in polychaetes, low diffusion distances between the external seawater and the blood, and a branchial circulatory system with a complexity comparable to that of the fish gill (Jouin and Gaill, 1990).

Hbs with high O_2 affinity can also be advantageous to species living in a poorly oxygenated environment (Weber, 1980). But what do the alvinellids actually breathe? According to Desbruyères *et al.* (1982) and Arp and Childress (*in* Terwilliger and Terwilliger, 1984), a mild to warm (up to 50°C), hypoxic water: *i.e.*, a mixture of the very hot, anoxic vent water and the cold, oxygenated local bottom seawater. But the oxygen concentration of this water mix has never been directly measured *in situ*, and the only direct evidence for low O_2 concentrations inside and outside hydrothermal vent community come from the Rose Garden vent field in the Galapagos Rift (Johnson *et al.*, 1986), where the O_2 content is always below ⅓ of the saturation at one atmosphere hydrostatic pressure. However, alvinellids have never been seen at the Rose Garden site, and the conditions there are quite different from those at the 13°N site. The hypothesis that alvinellids breathe hypoxic water must be considered, but is as yet not really supported.

Table V

Heat of oxygenation, $\Delta H = 2.303R\Delta \log P_{50}/\Delta(T^{-1})$, kJ/mol O_2. Mean values calculated between 10 and 40°C, for 4 pH values, 6.6 to 7.6

	6.6	6.9	7.25	7.6
Alvinella	−66	−76	−89	−102
pompejana	−0.976*	−0.990	−0.998	−0.999
Alvinella	−57	−63	−69	−76
caudata	−0.990	−0.998	−0.999	−0.995
Arenicola	−23	−23	−24	−24
marina	−0.972	−0.977	−0.987	−0.993

* Correlation coefficent.

The high O_2 affinity of alvinellid Hbs is modulated by the very large Bohr effect we found. The magnitude of the Bohr effect is extremely dependent on the oxygenation of the Hb molecule (Table IV): it is maximum when the molecule, almost fully deoxygenated, is in the so-called T-state (S_{O_2} *ca* 0%); minimum or null when the molecule, almost completely oxygenated, is in the R-state (S_{O_2} *ca* 100%); and intermediate when the molecule is half-oxygenated at P_{50}. These S_{O_2}-dependent Bohr-effect variations must greatly facilitate the O_2 unloading of the pigment at the tissue level, an advantage in view of the very high intrinsic O_2 affinity of alvinellid Hbs. The Bohr effect of the lugworm Hb is not as strong, and the maximum values of the Bohr factor occur when the Hb is half or nearly completely oxygenated, a property that Weber (1981) sees as favoring the O_2 loading of the pigment at the gill.

The oxygen transport efficiency of a respiratory pigment also depends on its cooperativity because, *in vivo*, a maximal cooperativity allows a maximal O_2 loading or unloading of the molecule for a corresponding minimal change of blood P_{O_2}. In alvinellid as well as in *Arenicola* Hbs, the O_2-binding process is highly cooperative, with n_{max} values that can be above 4 in *A. pompejana*. The value of n_{max} varies much more with temperature and pH in alvinellid than in lugworm Hbs (Fig. 6). In *Arenicola*,

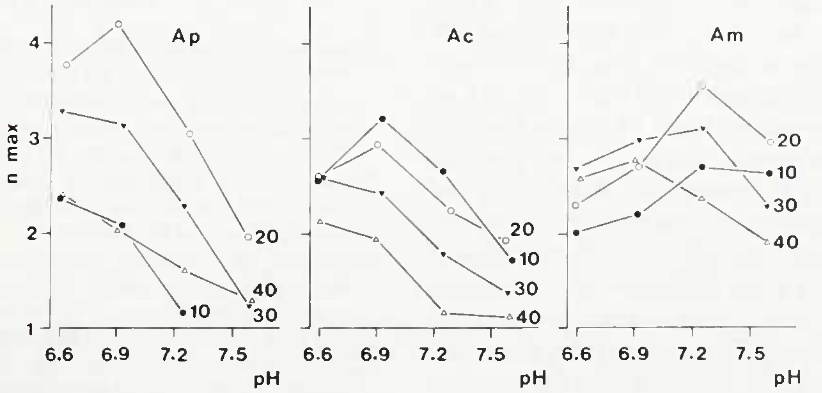

Figure 6. Variations of the Hill coefficient, n_{max}, as a function of pH at 10, 20, 30 and 40°C. Other experimental conditions: see text. (Ap) *Alvinella pompejana;* (Ac) *A. caudata;* (Am) *Arenicola marina*

which normally lives in cold to temperate waters (Wells, 1963), the physiological blood pH is 7.25 and 7.58 in animals acclimated at 26 and 5°C, respectively (Toulmond, 1977). The cooperativity of lugworm Hb is maximum between pH 7.25 and 7.6, and for temperatures between 10 and 30°C (Fig. 6). If the maximum cooperativity of the respiratory pigment is correlated with the physiological pH value in *Alvinella*, as it is in *Arenicola*, then the physiological range of blood pH in *Alvinella* is probably 6.6–6.9 and, in this pH range, the maximum cooperativity is obtained at 10–20°C in *A. caudata*, and at 20–30°C in *A. pompejana*. This blood pH range is unusually low for annelids and only direct measurements of blood pH could validate our interpretation of the data. But it is noteworthy that in the vestimentiferan worm *Riftia pachyptila*, another hydrothermal vent dweller living in sulfide-rich water, similar slightly acidotic pH values have been measured *in vivo* (Childress *et al.*, 1984).

Our observations suggest two sets of hypotheses: (i) the two alvinellid species form sympatric mixed populations on the same white or black smokers, but the external microenvironment is probably slightly colder for *A. caudata*, at 10 to 20°C, than for *A. pompejana*, at 20 to 30°C. These temperatures are well below the maximum temperature, 50°C, that the animals are supposed to withstand *in situ*. Our findings would corroborate Terwilliger and Terwilliger's (1984) observation that *A. pompejana* Hb is unstable at such a high, and probably nonphysiological, temperature. (ii) At those pH and temperature values, the *in vitro* intrinsic O_2 affinities of the Hbs are finally not so high, with P_{50} values ranging from 1.0 to 3.9 mm Hg in *A. pompejana* and from 0.4 to 1.8 mm Hg in *A. caudata* (Table III). The characteristics of the Bohr effect in these animals make such values quite compatible with an *in vivo* O_2 transport function for the Hbs. The additional hypothesis of Terwilliger and Terwilliger (1984), that the greater the depth, the greater the drop in the Hb O_2 affinity caused by hydrostatic pressure, then becomes unnecessary.

The lugworm and alvinellid Hbs differ by another characteristic. In the lugworm, the apparent heat of oxygenation, ΔH, is quite low, about −25 kJ/mol, and pH-independent. By contrast, in alvinellid Hbs, ΔH is strongly pH-dependent and is about three times higher, at pH 6.6–6.9, than in the lugworm Hb (Table V). These high ΔH values explain the important effects of a temperature change on the intrinsic O_2 affinity, the Bohr effect, and the cooperativity. A general inverse relationship can be established between the value of ΔH and the range of temperatures at which a given respiratory pigment has to function *in vivo*: the larger the temperature range, the lower the value of ΔH (see Toulmond, 1985, for examples). Since ΔH is higher for alvinellid than for lugworm Hbs, the alvinellids probably live in an environment better temperature-regulated than that of the intertidal lugworm. This conclusion might seem to be inconsistent with the supposed extreme environmental variability around the hydrothermal vents, but annelids, and especially those living in elaborate tubes or galleries, are capable of creating their own regulated microenvironment (Toulmond, 1990). But as for O_2 concentrations, *in situ* direct measurements of the temperature microdistributions inside and outside the alvinellid tubes are needed.

In conclusion, although the alvinellid Hbs are structurally very similar to those of annelids living in more ordinary habitats, these Hbs clearly exhibit some distinct functional properties that are most probably directly related to the characteristics of the hydrothermal vent environment. Their properties suggest that the alvinellid Hbs function as O_2 carriers at slightly acidic blood pH values and at fairly constant temperatures, not exceeding 20°C for *A. caudata* and 30°C for *A. pompejana*. This could indicate that both species can create a differentiated and stable external microenvironment.

Acknowledgments

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The Efflux of Amino Acids from the Olfactory Organ of the Spiny Lobster: Biochemical Measurements and Physiological Effects

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Abstract. The amino acids taurine and glycine are odorants that activate specific chemosensory cells in the olfactory sensilla (aesthetascs) of the spiny lobster, *Panulirus argus*. We show that the aesthetascs themselves contain large intracellular concentrations of taurine (≈ 2 mM) and glycine (≈ 85 mM); these concentrations are more than 10,000-fold greater than the response thresholds of the chemosensory cells. A net efflux of at least five amino acids occurs when the olfactory organ is immersed in amino acid-free seawater. With taurine and glycine, efflux continues until an apparent equilibrium is reached between the sensilla and the external medium; for taurine the equilibrium with seawater occurs at ≈ 12 to 28 nM, and for glycine at ≈ 100 to 500 nM. Aesthetascs may achieve these equilibria within 300 ms. Hence, even during the brief interval between consecutive flicks of the antennule, olfactory receptors are exposed to a background of odorants escaping from intracellular stores. Electrophysiological studies show that both the spontaneous and evoked activities of taurine-sensitive chemosensory cells are markedly affected by a taurine background simulating that measured in the efflux studies. Uptake systems may participate in establishing the equilibria between sensilla and seawater since (1) the net efflux of amino acids increases in sodium-free seawater; and (2) guanidinoethane sulfonate, a competitor for taurine uptake, selectively increases net taurine efflux. Effluxes from an olfactory organ may contribute noise to the chemosensory process; alternatively, background substances could contribute functionally by affecting membrane proteins.

Introduction

Olfactory sensilla on the antennules of the Florida spiny lobster, *Panulirus argus*, contain populations of chemoreceptor cells with differential specificities for taurine, glycine, and other amino acids (see review by Carr *et al.*, 1987). Physiological studies have revealed that taurine-sensitive cells have response thresholds of about 10^{-10} M (Fuzessery *et al.*, 1978; Ache *et al.*, 1988), with some cells being activated by taurine at concentrations as low as 10^{-12} M (Thompson and Ache, 1980). The response thresholds of the glycine-sensitive cells ($\approx 10^{-6}$ M; Ache *et al.*, 1988) are generally higher than those of the taurine-sensitive cells. Chemoreceptors sensitive to exogenous amino acids are not unique to the spiny lobster. Indeed, chemoreceptor cells with selective sensitivities to specific amino acids also occur in several other crustaceans including the American lobster, *Homarus americanus* (Derby and Atema, 1982; Johnson and Atema, 1983), a crayfish, *Austropotamobius torrentium* (Hatt, 1984), a prawn, *Macrobrachium rosenbergii* (Derby and Harpaz, 1988), and a crab, *Carcinus maenas* (Schmidt and Gnatzy, 1989).

In addition to serving as exogenous chemoexcitants of crustaceans, amino acids such as taurine and glycine also occur intracellularly in very high concentrations (10^{-1} to 10^{-3} M), and contribute to osmotic regulatory processes (*e.g.*, Yancey *et al.*, 1982; Pierce, 1982). The antennular nerve of the spiny lobster, for example, contains taurine at a concentration of about 4 mM (see Results). Thus, this animal maintains an intracellular concentration of taurine that is more than a million-fold higher than the

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Abbreviations: ASW: artificial seawater; GES: guanidinoethane sulfonate; HPLC: high performance liquid chromatography; OPA: *ortho*-pthaldialdehyde.

response thresholds reported for its taurine-sensitive chemoreceptors. If the sensory cells themselves contain millimolar concentrations of taurine, then a small leakage from these cells into the receptor environment could generate a high background level of taurine that might negate the apparent utility of receptors with nanomolar sensitivities. The leakage of internal chemicals into the receptor environment has indeed been implicated in mammals, where chemostimulants injected into the blood stream activated olfactory (Maruniak *et al.*, 1983) and gustatory receptors (Bradley and Mistretta, 1971).

In the present study, we show that the olfactory sensilla of the spiny lobster contain high intracellular concentrations of free amino acids. Using an attached intact antennular preparation (Trapido-Rosenthal *et al.*, 1990), we show that there is a measurable efflux of at least five amino acids from the olfactory organ. Although the efflux of each amino acid appears to be regulated, the efflux of glycine is most pronounced and may produce glycine backgrounds of up to 5×10^{-7} *M* in the receptor environment. Finally, we demonstrate that the physiological responses of taurine-sensitive cells can be affected by a taurine background of about 10^{-8} *M* which occurs because of the efflux from intracellular pools.

Materials and Methods

Collection and maintenance of animals

Specimens of the Florida spiny lobster, *Panulirus argus*, were collected in the Florida Keys and maintained at the Whitney Laboratory in flowing seawater on a diet of fish, squid, and shrimp. Only adult intermolt animals were used.

Biochemical procedures

Amino acid analysis. Amino acids present in tissue samples or in aliquots of incubation media were derivatized with *ortho*-pthaldialdehyde (OPA) (Lindroth and Mopper, 1979). The fluorescent derivatives were separated by HPLC; an octadecylsilane column packed with 4 μm beads (Waters Nova-Pak C_{18}) and eluted with Buffers A and B was used as described by Manahan (1989). Buffer A consisted of 50 m*M* sodium acetate (pH 6.8), methanol, and tetrahydrofuran (80:19:1). Buffer B consisted of 50 m*M* sodium acetate and methanol (20:80). Derivatized amino acids were eluted from the column according to a step gradient procedure modified from Manahan (1989), so that at 1, 6, 11, and 16 min after sample injection, the percentage of Buffer B was increased from 0 to 25, 50, 75 and 100, respectively. The flow rate was 1.2 ml per min. Fluorescent derivatives were detected with a Bio-Rad Model 1700 fluorometer fitted with a 360 nm excitation filter and a 440 nm emission filter; peaks were integrated by means of a Waters Model 730 Data Module. Identification and quantitation of amino acid derivatives were performed by comparisons with standards.

Extraction of amino acids from tissues. Olfactory sensilla (= aesthetascs) were collected from lateral antennular filaments after blotting, rapid freezing in liquid nitrogen, and lyophilization. The sensilla were removed with fine-tipped forceps, and their numbers estimated by counting the antennular segments harvested. Antennular nerve sections, approximately 2 cm in length, were dissected from lateral antennular filaments at a position just proximal to the aesthetasc tuft. Dissections were performed in a bath of *P. argus* saline, and the tissue blotted, weighed, and frozen. Hemolymph samples (1 ml) were withdrawn at the base of a walking leg; a chilled syringe was used to minimize clotting. Samples were immediately centrifuged (12,000 × *g*) to remove cellular material, and the supernatant frozen.

Free amino acids in tissue samples were extracted by homogenization in a solution of 80% methanol/20% sodium acetate (50 m*M*, pH 6.8) followed by centrifugation (16,000 × *g*). The supernatants were transferred to clean tubes, evaporated to dryness, then redissolved in 50 m*M* sodium acetate for reaction with OPA as described above.

Net efflux of amino acids from the aesthetasc sensilla of attached intact antennules. Lobsters were removed from the water and immobilized on racks as described previously (Trapido-Rosenthal *et al.*, 1990). The distal portions of the intact lateral antennular filaments were placed in vials containing 3.5 to 4.5 ml of artificial seawater (ASW; see Gleeson *et al.*, 1989) which was vigorously agitated by using magnetic stirring bars. At selected times, samples of the ASW (= incubation medium) were removed from the vials for amino acid analysis. The above procedure ensured that the only part of the lobster contacting the incubation medium was the antennular filament; all references to the use of intact antennules are references to this procedure.

In one experiment, analyses were performed on amino acids released into ASW by both aesthetasc-bearing and aesthetasc-free sections of antennular cuticle. These isolated sections were prepared as described in Trapido-Rosenthal *et al.* (1987).

In experiments to investigate the effect of low concentrations of sodium on amino acid efflux from the aesthetascs, intact antennules were subjected to three sequential, 10-min incubations. In the first incubation, the antennule was immersed in ASW; in the second incubation the antennule was immersed in artificial seawater in which the sodium chloride had been replaced with equimolar choline chloride. The third incubation was again performed in ASW. The effect of the taurine-uptake competitor, guanidinoethane sulfonate (GES), on amino acid efflux was examined according to a similar protocol except that the second of the three incubations contained 1 μM GES in ASW.

Electrophysiological procedures

The responses of single cells stimulated by taurine were recorded extracellularly from the isolated perfused lateral filament of the antennule. The olfactometer and recording procedures have been described in detail previously (Gleeson and Ache, 1985). Action potentials (impulses) from single cells were discerned via an amplitude/time window discriminator, and the time intervals between impulses analyzed with a microprocessor. In this report, cell responses are quantified in two ways: (1) the total number of impulses occurring within a 5- or 10-s period following onset of the response; and (2) maximum frequency, defined as the mean instantaneous frequency determined for the four shortest intervals between successive impulses.

Effects of background taurine. Cells stimulated by taurine were identified by introducing a 10-μM search stimulus into the carrier stream of ASW that continuously flowed past the olfactory sensilla at a rate of 3 ml/min. Once a taurine-sensitive cell had been identified, the dose-response function was determined in the presence and absence of an imposed background of 10 nM taurine. This background simulated a representative concentration present in the receptor environment as calculated from our measurements of the efflux of endogenous taurine (*vide infra*). The effect of the background on the dose-response function was determined by applying an ascending series of taurine concentrations; each concentration was tested with and without background before the next higher concentration in the series was applied. For tests in the presence of background, the carrier stream of ASW contained 10 nM taurine which flowed through the olfactometer for 2 min before and during the introduction of a test stimulus. The response to each concentration was monitored following the injection of 190 μl into the carrier flow of ASW. With this volume, the concentration of taurine in the olfactometer reached the injected level within 1 s and began to decline after a 2-s plateau period (Zimmer-Faust *et al.*, 1989). Following the presentation of each stimulus, the preparation was flushed with ASW for 2 min.

To determine whether taurine-sensitive cells are affected by taurine circulating in the hemolymph, cell responses were examined while the antennule was perfused with *P. argus* saline containing various concentrations of this amino acid. For these experiments, the responses to exogenous taurine injected into the carrier flow of ASW were compared in the presence and absence of taurine in the perfusion medium. For tests with taurine in the perfusion saline, cell responses were examined at 5-min intervals for up to 25 min.

Stimulus preparation. Taurine stock solutions (1 mM) were prepared in ASW, the pH adjusted to 7.8, and aliquots stored at −70°C. At the beginning of each experiment, test stimuli were prepared by serially diluting the stock solution with ASW. For stimuli presented in the presence of a taurine background, stock solutions were serially diluted in ASW containing 10 nM taurine.

Reagents

Amino acids and the fluorescent derivatizing reagent *ortho*-pthaldialdehyde (OPA) were purchased from Sigma Chemical Company. Guanidinoethane sulfonate (GES) was generously provided by Dr. Ryan Huxtable. Reagent-grade salts were from Fisher Scientific Company, as were HPLC-grade methanol and tetrahydrofuran.

Results

Amino acid content of the aesthetasc sensilla

Aesthetascs on the lateral filament of the antennule contain high intracellular concentrations of several free amino acids. A chromatogram of amino acids extracted from aesthetascs appears in Figure 1; intracellular concentrations of five of these amino acids are shown in Figure 2. Glycine occurs in the sensilla at a concentration of 85.2 ± 2.4 mM and is the predominant OPA-derivatizable amino acid in the intracellular pool. Taurine is a far more minor constituent, occurring at a concentration of 2.1 ± 0.7 mM.

The composition of free amino acids in the aesthetascs differs markedly from that of both the antennular nerve and the hemolymph (Fig. 2). For example, glycine is the major component within both the aesthetascs and hemolymph, but is superseded by aspartate in antennular nerve; taurine, which is a relatively minor constituent in the aesthetascs and antennular nerve, occurs at a mid-range concentration within the hemolymph. In addition to these differences in relative concentrations between tissues, the absolute concentrations of the amino acids in hemolymph are considerably lower than those within the aesthetascs and antennular nerve.

Amino acid efflux from the aesthetasc sensilla

When intact antennules are placed into amino acid-free ASW, a net efflux of amino acids occurs (Fig. 3). During 10-min incubations, glycine efflux ranged from 51.5 to 299.9 fmoles per sensillum, whereas taurine efflux ranged from below the limits of detection by OPA to 29.5 fmoles per sensillum. We assumed that the aesthetascs are the major sites of amino acid efflux based upon the following considerations. (1) The aesthetascs on each antennule contain approximately 12.5 cm^2 of dendritic membrane which is separated from the external seawater

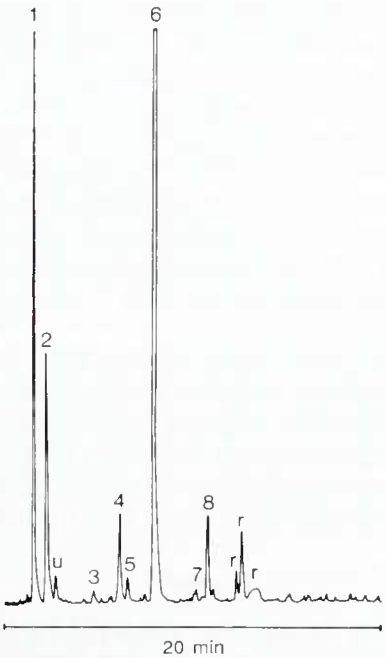

Figure 1. HPLC chromatogram of free amino acids extracted from 90 aesthetasc sensilla from a single antennule. 1 = aspartic acid (92 pmoles); 2 = glutamic acid (41 pmoles); 3 = asparagine (7 pmoles); 4 = serine (18 pmoles); 5 = histidine (16 pmoles); 6 = glycine (370 pmoles); 7 = taurine (4 pmoles); 8 = alanine (17 pmoles); r = reagent peaks; u = unknown.

environment by only a thin layer of permeable cuticle (surface area calculated from the data of Grünert and Ache, 1988). This surface area far exceeds the cell-membrane area exposed by other cuticular structures on the lateral filament of the antennule. (2) The concentration profiles of amino acids present within the aesthetascs and in the efflux from the antennule are markedly similar (Inset, Fig. 3). (3) The amino acid effluxes from aesthetasc-bearing and aesthetasc-free sections of antennular cuticle were compared, and the efflux from the former exceeded the latter by 25-fold. Sensilla-associated microorganisms may have made positive or negative contributions to the measured effluxes, but neither light nor electron microscopy have revealed extensive populations of such organisms (R. A. Gleeson, pers. comm.).

Amino acid transport in invertebrates, like that of vertebrates, is a Na^+-dependent process (*e.g.*, Gunn, 1980; Stevens *et al.*, 1984; Wright and Pajor, 1989). The Na^+-dependence of these transport systems is presumed to represent a carrier-mediated co-transport of Na^+ in which the energy is provided by the transmembrane Na^+ gradient. Incubation of antennules in Na^+-free ASW caused a reversible increase in the net efflux of amino acids (Fig. 4). The enhancement of efflux was greater than twofold for each amino acid measured and was restored to control levels when the antennules were returned to Na^+-containing ASW. This enhancement was significant for each of the amino acids with the exception of taurine [Repeated Measures ANOVA coupled with a Planned Comparisons Test (CSS, StatSoft): $P < 0.05$, $n = 3$]. The data for taurine did, however, approach significance ($P = 0.06$).

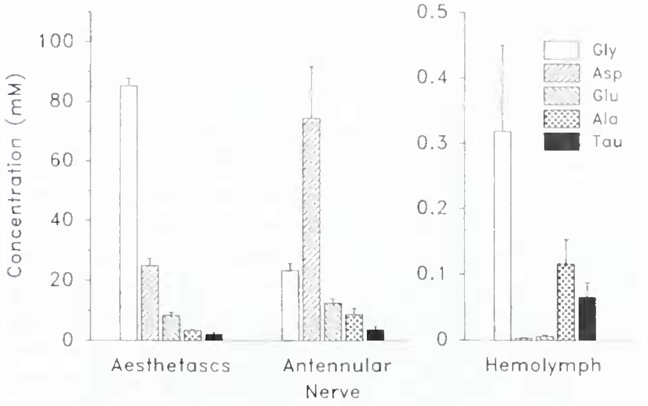

Figure 2. Mean concentrations (+SEM) of selected free amino acids in aesthetasc sensilla, antennular nerve and hemolymph of the spiny lobster. Intracellular concentrations for aesthetascs are based on the assumption that cells occupy 20% of the 250 pl volume of each sensillum (calculated from Grünert and Ache, 1988). For determining antennular nerve concentrations, tissue wet weights were converted to volume assuming a specific gravity of unity. Tissues from six animals were used in these determinations, except that the aesthetasc sensilla from the antennules of four animals were used. Gly = glycine; Asp = aspartic acid; Glu = glutamic acid; Ala = alanine; Tau = taurine.

The net efflux of taurine from antennules showed about a five-fold enhancement in the presence of 1 μM GES, a specific competitor for taurine and other substances internalized by β-amino acid transport systems (Huxtable *et al.*, 1979; Quesada *et al.*, 1984) (Fig. 5). The enhancement of taurine efflux was significant [Repeated Measures

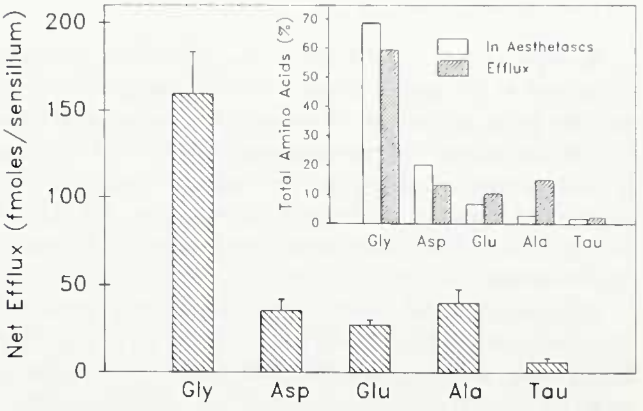

Figure 3. Net efflux of amino acids from intact antennules during 10-min incubations in ASW. Values are the means + SEM for 14 animals. Inset compares the profiles of amino acids in the aesthetascs (as shown in Fig. 2) with those released into the ASW. Amino acid abbreviations as in Figure 2.

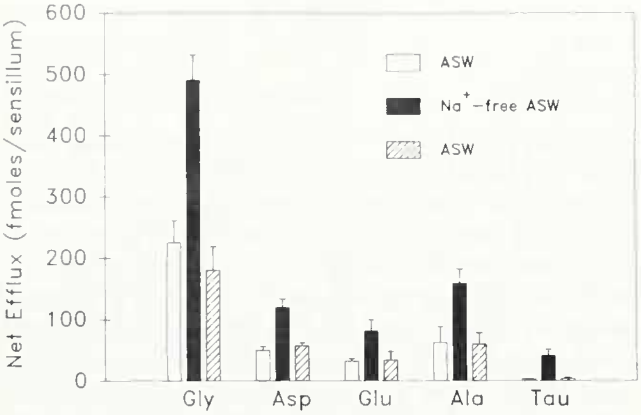

Figure 4. Efflux of amino acids from intact antennules incubated sequentially in ASW (open bars), Na$^+$-free ASW (solid bars), and ASW (hatched bars). In Na$^+$-free ASW, NaCl was replaced with equimolar choline chloride. Incubations were for 10 min. Values are the means + SEM of three experiments. Amino acid abbreviations as in Figure 2.

ANOVA coupled with a Planned Comparison Test (CSS, StatSoft): $P < 0.05$, n = 3] and reversible. GES did not significantly affect the efflux of the other amino acids examined.

The time course for the appearance of glycine and taurine in the incubation medium was determined for the antennules of three different animals (Fig. 6). Although considerable inter-animal variability existed, in each case and for both amino acids, the extracellular concentration increased until an apparent equilibrium concentration was attained. The equilibrium concentrations ranged from 100 to 500 n*M* for glycine (Fig. 6A) and from 12 to 28 n*M* for taurine (Fig. 6B). The occurrence of a rapid efflux to an equilibrium concentration was typically observed for other amino acids as well.

Physiological effects of a taurine background

In the presence of a 10 n*M* background concentration of taurine in the carrier stream of ASW, the spontaneous activity in all of the taurine-sensitive cells examined obviously increased. This increase was significant; following a 2-min exposure to the taurine background, the mean number of impulses per second was 4.26 ± 0.28 (±SEM) *versus* 0.88 ± 0.08 in the absence of taurine (Wilcoxon Signed Rank Test: $P < 0.01$, n = 9).

The dose-response function for injected (= exogenous) taurine in the presence and absence of the 10 n*M* taurine background is shown in Figure 7. Expressing the response in terms of either maximum frequency or impulses per 5 s reveals a dose-dependent increase in activity which appears to attain a maximum level between 3.3 and 10 μM taurine. In the presence of the taurine background, there is an apparent downward shift in the dose-response function. This shift is highly significant as revealed by the intercept differences for the linear regions (*i.e.*, between 0.01 and 3.3 μM taurine) of the maximum frequency curves [Random Coefficient Regression Analysis with intercepts compared using a Wilcoxon Signed Rank Test: $P = 0.004$, n = 9]. A paired comparison of the maximum responses (*i.e.*, responses to 10 and 33 μM taurine) for cells in the presence and absence of background taurine also yielded a significant difference [Wilcoxon Signed Rank Test: $P = 0.03$ (maximum frequency data), $P < 0.001$ (impulses per 5 s data), n = 15].

The responses of taurine-sensitive cells to exogenous taurine stimuli were unaffected by the presence of taurine in the perfusion saline (Fig. 8). For the six cells examined, the mean responses to test stimuli in the presence and absence of taurine in the perfusion medium were virtually identical (Wilcoxon Signed Rank Test: $P = 0.438$, n = 6).

Discussion

Cells within the aesthetasc sensilla of the spiny lobster contain the amino acids glycine and taurine at concentrations of about 85 and 2 m*M*, respectively. Following immersion of the intact lateral antennular filament in amino acid-free seawater, a net efflux of glycine and taurine from the sensilla occurs until an apparent equilibrium is reached with the external medium; for glycine the equilibrium concentration in the seawater is about 100 to 500 n*M*, and for taurine it is approximately 12 to 28 n*M* (Fig. 6). The establishment of these equilibria indicates that aesthetascs can maintain intracellular glycine and taurine at concentrations that are more than 100,000-fold greater than those in the external medium. However, the existence of apparent limits on the ratio of intracellular to extracellular concentrations suggests that, even in seawater free

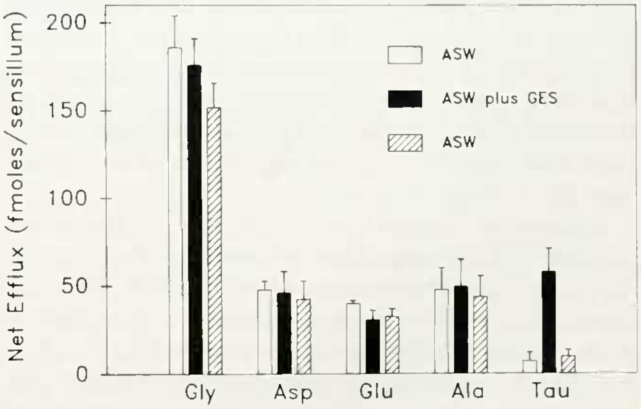

Figure 5. Efflux of amino acids from intact antennules incubated sequentially in ASW (open bars), ASW containing 1 μM GES (solid bars), and ASW (hatched bars). Incubations were for 10 min. Values are the means + SEM of three experiments. Amino acid abbreviations as in Figure 2.

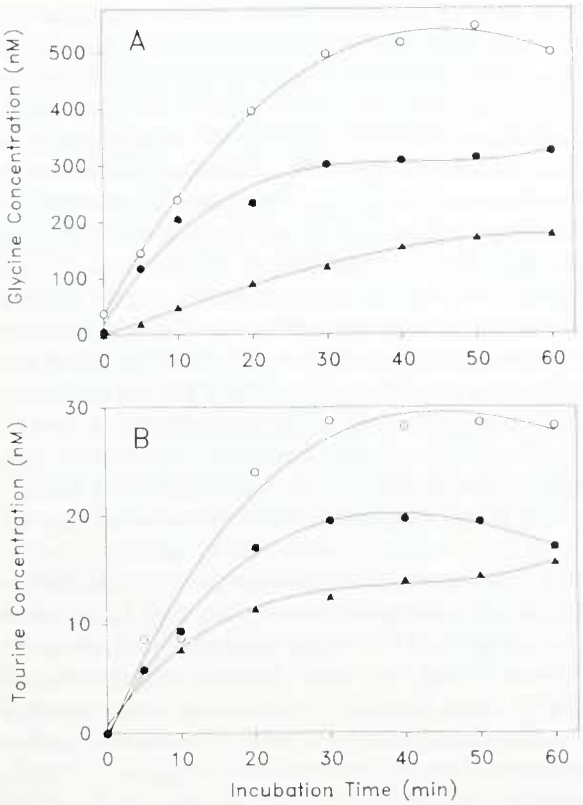

Figure 6. The time course of efflux for glycine (A) and taurine (B) from intact antennules of three animals. At each time point, 100-μl aliquots were removed from the incubation vials and the amino acid concentrations in the incubation media were determined. Apparent equilibrium concentrations were attained in the 4.5-ml incubation volumes within 30 to 40 min.

of exogenous glycine or taurine, sensillar receptors will be exposed to background ("noise") levels of these amino acids because of their efflux from intracellular pools.

The effluxes of glycine and taurine from the olfactory organ create, at equilibrium, background levels that correlate quite well with those occurring in natural seawater. Glycine is frequently present in seawater at levels approaching 100 n*M* or greater (*e.g.*, Garrasi *et al.*, 1979; Braven *et al.*, 1984; Siebers and Winkler, 1984), whereas taurine is often not detected and seldom exceeds 10 n*M* (Mopper and Lindroth, 1982; Wright and Secomb, 1986). The existence of a low background (*i.e.*, low noise) level of taurine in seawater, plus the occurrence of only a slight efflux from the olfactory organ, suggests that taurine leaking from a prey organism would be more readily detected than glycine. For taurine, an effective signal-to-noise ratio could exist at exogenous concentrations above approximately 10 n*M*; whereas for glycine, good signal-to-noise ratios would require concentrations greater than about 100 n*M*. Indeed, the contrasts between the low (nanomolar) thresholds of taurine-sensitive cells (Fuzessery *et al.*, 1978; Ache *et al.*, 1988), and the apparently higher (micromolar) thresholds of glycine-sensitive cells (Ache *et al.*, 1988), may be expressions of receptor adaptations to the exigencies of different background concentrations.

The lobster obtains discontinuous samples of its chemical environment by periodically flicking its antennules in a manner that rapidly exchanges water trapped between the densely arranged aesthetasc sensilla (Price and Ache, 1977; Schmitt and Ache, 1979; Moore and Atema, 1988; R. A. Gleeson, pers. comm.). The time interval between successive flicks (interflick interval) can vary from about 500 ms to over 30 s (R. A. Gleeson, pers. comm.). During this interflick period, seawater trapped between the aesthetascs forms a large boundary layer within which odorant movement is essentially restricted to molecular diffusion (Schmitt and Ache, 1979; Moore and Atema, 1988). As a consequence, chemoreceptors within the aesthetascs are primarily exposed to whatever odorants are captured during the preceding flick, with the actual concentrations at the receptors being dependent upon the rates of odorant diffusion between the aesthetascs and the

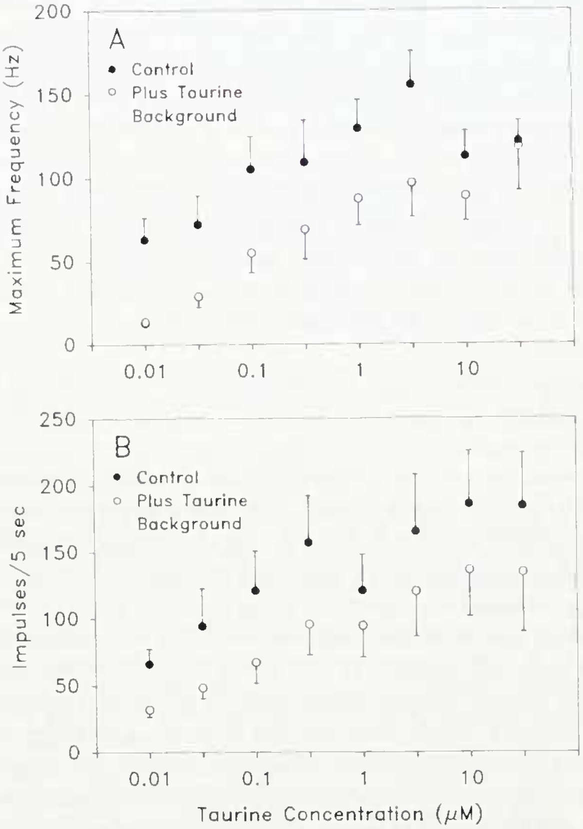

Figure 7. Dose-response functions for taurine-sensitive cells in the presence and absence of a 10 n*M* taurine background. Response magnitude is expressed in terms of maximum frequency (A) and total number of impulses during the first 5 s of the response (B). Points are the means ± SEM for nine cells.

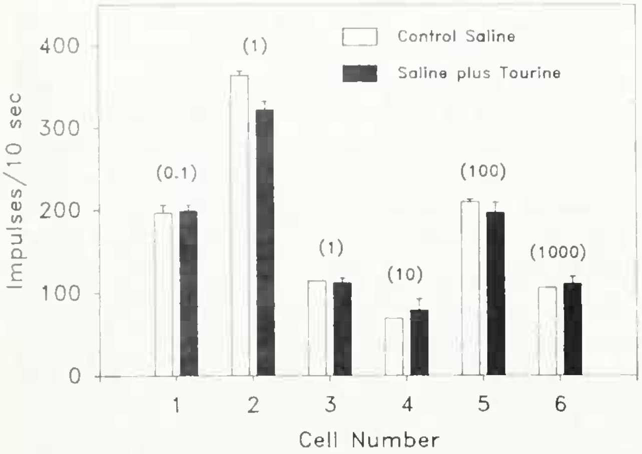

Figure 8. Mean responses (+SEM) of taurine-sensitive cells in the presence and absence of taurine in the perfusion saline. The micromolar concentrations of taurine in the perfusion medium are indicated in parentheses. For each cell, a test-stimulus concentration close to the EC_{50} was presented via the carrier stream of ASW.

boundary layer of seawater. This boundary layer can also limit the rate of amino acid efflux from aesthetascs by acting as a buffer between the sensilla and seawater outside the aesthetasc tuft. If it is assumed that a major fraction of the glycine and taurine leaking from the cells of an aesthetasc during the interflick interval remains within the sensillar lymph (volume $\approx$ 200 pl), then only about 300 ms would be required for the sensillum to attain the equilibrium concentrations measured in the current study (Fig. 9). Hence, during a considerable portion of each interflick interval, the sensillar receptors are probably exposed to backgrounds ranging from low nanomolar in the case of taurine, to as high as 0.5 micromolar in the case of glycine.

When sensilla are immersed in seawater, the efflux of glycine and taurine from intracellular stores does not continue unabated until the concentration in the sensilla and medium are equal. Rather, at equilibrium the intracellular concentration is about 100,000-fold greater than the medium. Uptake systems in the sensilla are the most plausible mechanisms for regulating the amino acid efflux. The hypothesis that uptake systems might control the net efflux or loss of intracellular amino acids was proposed by Wright and Secomb (1986) based on studies with the gills of marine mussels. These workers noted that mussel gills contain intracellular taurine at a concentration of about 60 m*M*, and that a net efflux occurred into seawater. They demonstrated the existence of a taurine uptake system and showed that it was able to recapture up to 30% of the taurine escaping from the gills. They then proposed that this re-uptake conserves energy and contributes to maintaining the high intracellular concentrations of taurine (Wright and Secomb, 1986; Wright, 1987). Our study on amino acid efflux from the lobster olfactory organ reveals the following parallels with these findings from molluscan gills: (1) immersion in amino acid-free seawater results in a net efflux of amino acids that continues until an apparent equilibrium is established with the external medium (Fig. 6); (2) the net efflux of amino acids increases in Na^+-free seawater (Fig. 4); and (3) a selective competitor of taurine uptake increases the net taurine efflux (Fig. 5) (Wright and Secomb, 1984, 1986; Wright *et al.*, 1989). Regarding the olfactory organ of the lobster, we already know that uptake systems for taurine and other amino acids are present in the olfactory sensilla (Gleeson *et al.*, 1987; Trapido-Rosenthal *et al.*, 1988). However, the kinetics of uptake exhibited by the excised sensilla used in these earlier studies are not compatible with maintaining the equilibrium concentrations measured in the current study. The intact antennular preparation should now be employed to re-examine the kinetics of uptake.

In the American lobster, *Homarus americanus*, the adaptation of NH_4-sensitive chemosensory cells to increased background levels of NH_4 was studied in detail by Borroni and Atema (1988). For each imposed background, adaptation of these receptor cells included: (1) a re-setting of the response threshold to an NH_4 concentration greater than background; and (2) a concomitant, parallel rightward shift in the stimulus-response function (*ibid.*). In the present study, the exposure of taurine-sensitive cells of the spiny lobster, *P. argus*, to a background level of taurine yielded results having both similarities and differences to those described for the NH_4-cells of *H. americanus*. Unlike

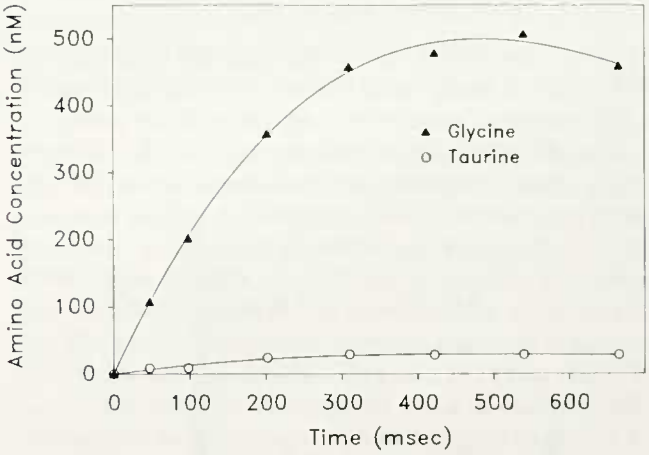

Figure 9. Calculated concentrations for glycine and taurine in the sensillar lymph of a single sensillum from the animal represented by open circles in Figure 6. Assumptions used were as follows. (1) At t_0, the antennule is flicked and the 200-pl extracellular volume within the sensillum immediately equilibrates with the amino acid-free seawater. (2) During the subsequent interflick interval, all amino acids released from the cells of the sensillum are retained in the 200-pl volume of sensillar lymph. Under these conditions, both glycine and taurine attain their equilibrium concentrations in the lymph within 300 ms.

the responses of NH_4-cells to NH_4 backgrounds, taurine-sensitive cells did not exhibit complete adaptation to the taurine background examined. Instead, after an initial phasic response when the background was first introduced, these cells reached a new tonic level of activity that was significantly greater than their spontaneous activity in ASW. As in NH_4-cells, adaptation (albeit partial) was indicated by a rightward, or seemingly downward, shift in the dose-response function. In the taurine-sensitive cells, this shift included an apparent reduction in the maximum response; this effect might be considered functionally equivalent to either a generalized reduction in the efficacy of taurine, or to an inactivation of some proportion of the receptor population.

In the current study, the responses of taurine-sensitive chemosensory cells in the antennule were not affected when taurine concentrations as high as 1 m*M* were presented internally via the perfusion saline (Fig. 8). These results imply that a functional barrier, at least for taurine, separates the hemolymph and the sensillar lymph. These results contrast with findings in mammals where certain chemostimulants injected into the blood stream were found to stimulate olfactory (Maruniak *et al.*, 1983) and gustatory receptors (Bradley and Mistretta, 1971).

A high intracellular concentration of low molecular weight organic substances is a characteristic feature of organisms subjected to water stresses, including high or fluctuating salinity (Yancey *et al.*, 1982). These organic substances together with inorganic ions represent the major osmotically active solutes (osmolytes) present within all cells. Prominent among these compounds are certain amino acids including taurine, glutamate, alanine, glycine, proline, and aspartate (Clark, 1985). The similarities between species in the chemical properties of organic osmolytes are remarkable; these properties parallel those of cations and anions in the Hofmeister series that favor compatibility with protein structure and function. Indeed, these intracellular substances may be important in offsetting the destabilizing or perturbing effects that high neutral salt concentrations have on macromolecules (Yancey *et al.*, 1982). By analogy with the intracellular effects described above, the efflux of certain amino acids (*e.g.*, glycine) and other osmolytes into the receptor environment within olfactory sensilla may play a general role in stabilizing the extracellular domains of various membrane proteins associated with the sensory dendrites. The efflux of such compounds could be important in maintaining the structure and function of receptors, channel proteins, transporters and ecto-enzymes that would otherwise be directly exposed to seawater. Indeed, certain of these substances may specifically modulate the activity of some membrane proteins. For example, extracellular glycine occurring in synaptic clefts binds to specific sites on the NMDA-glutamate receptor-channel complex and contributes significantly to receptor activation and channel function (*e.g.*, Kessler *et al.*, 1989; Thomson, 1989). Organic osmolytes in the extracellular lymph of olfactory sensilla may play similar regulatory roles in chemosensory processes.

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INDEX

A

Abrahamse, S. L., see Janine L. Kallen, 351
Abramson, Charles I., see Richard D. Feinman, 233
Abstracts of the MBL General Meetings, 219–235
Actin, 219, 220
Action spectrum, 340
Activity rhythm, 178
Aequorin, 224
Aerial fields, 225
Aerial vibrating probe, An, 225
Aiptasia pallida, 148
Akiyama, T., and M. Yoshida, The nocturnal emergence activity rhythm in the cumacean *Dimorphostylis asiatica* (Crustacea), 178
Alexander, David, Drag coefficients of swimming animals: effects of using different reference areas, 186
Allorecognition in sponge, 272
Alpha-2 polyglobulin, 233
Alvinella, 366
Alvinellid extracellular hemoglobins, 366
Amano, Shigetoyo, Self and non-self recognition in a calcareous sponge, *Leucandra abratsbo,* 272
Amino acid efflux and uptake, 374
Ammonia induces settlement behavior in oyster larvae, 297
Amoebocyte, 219
Ampullae, 220
Anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae), The, 332
Annelids, hydrothermal vent, 366
Anthozoa, 148
Anti-AIDS agent, 226
Anti-fertility agent, 226
Anticarcinogens, 221
Antimalarial drugs block calcium currents in *Paramecium,* 228
Antipathes fiordensis, 96
Aplysia, 229
Aplysia ink, 326
Application of a magnetic current probe to map axial inhomogeneities in a squid giant axon, 229
Arbacia development, 221
Armstrong, P., see J. Quigley, 221
Armstrong, Peter B., Walter F. Mangel, Atsushi Ikai, Kensal E. Van Holde, and James P. Quigley, Structure of alpha-2 macroglobulin from the horseshoe crab, 233
Arnold, John M., Hyla C. Sweet, and Barbara C. Boyer, S.E.M. observations of early cleavage in *Hoploplana inquilina,* 220
Artemia, 287
Ascidian, 140
  ampullae, 220
  development, 222
Associations between egg capsule morphology and predation among populations of the marine gastropod, *Nucella emarginata,* 312
Asterias vulgaris, 159
Asteroidea, 159, 254
Atema, Jelle, and Paul A. Moore, Boundary layers and microscale fluid dynamics around the lobster's (*Homarus americanus*) chemosensory appendages, 234
Atema, Jelle, see Adele Pile, 234; George Gomez, 234; H. F. Gerardo, 234; and Nat Scholz, 235
Auricularia, 121
Axonal transport, 229
Ayvazian, Suzanne G., see Robert Billard, 222; Heidi E. Geyer, 223; Kristen M. O'Brien, 223; and Rebeka J. Rand, 223

B

Baker, R., see E. Gilland, 230
Ballantyne, James S., see John A. Berges, 287
Barlow, Robert Jr., see Erik Herzog, 230
Barry, Susan R., Juan Bernal, and Barbara E. Ehrlich, Antimalarial drugs block calcium currents in *Paramecium,* 228
Bates, William R., Morphogenesis of ascidian ampullae and polarized movements of tunic extracellular matrix components along ampullae, 220
Bearer, E. L., M. Strout, and David Demers, Detailed observations on the migration of *Limulus* amoebocytes by video microscopy, 219
Behavior, 87
Bendena, W. G., see M. L. Pardue, 77
Bennett, M. V. L., see C. D. Leidigh, 224; and D. C. Spray, 225
Berges, John A., John C. Roff, and James S. Ballantyne, Relationship between body size, growth rate, and maximal enzyme activity in the brine shrimp, *Artemia franciscana,* 287
Berghia, 243
Bernal, Juan, see Susan R. Barry, 228
Berns, Donald S., see Robert MacColl, 326
Bezprozvanny, Ilya B., James Watras, and Barbara E. Ehrlich, Calcium-dependence of inositol 1,4,5-trisphosphate-gated calcium channels from endoplasmatic reticulum of cerebellum is bell-shaped, 228
Bilins, 326
Biliproteins, 326
Billard, Robert, Laura Lynch, Linda A. Deegan, John T. Finn, and Suzanne G. Ayvazian, Feeding and predation rates of winter flounder, *Pseudopleuronectes americanus,* and blue crabs, 222
Binding substance, 140
Bioassay, 227
Biochemical and functional effects of sulfate restriction in the marine sponge, *Microciona prolifera,* 358
Bioluminescence, 87, 340
Bipinnaria, 121
Black coral sweeper tentacles, 96
Blood cells, 140
Blood pressure, 233
Blue crab, 191, 222
Bonar, D. B., see S. L. Coon, 297
Boundary layers and microscale fluid dynamics around the lobster's (*Homarus americanus*) chemosensory appendages, 234
Bowlby, Mark R., Edith A. Widder, and James F. Case, Patterns of stimulated bioluminescence in two pyrosomes (Tunicata: Pyrosomatidae), 340
Boyer, Barbara C., Macromere control of early development in the polyclad flatworm, *Hoploplana,* 221
Boyer, Barbara C., see Hyla C. Sweet, 222; Ilene M. Kaplan, 227; and John M. Arnold, 220
Boyer, John F., Stochastic and deterministic models of niche displacement, 223
Braithwaite, R. S., see J. Quigley, 221

Buccal, 229
Buck, John, Unisex flash controls in dialog fireflies, 87, (3)vi
Bullhead catfish, 234
Bullis, Robert A., Submersible vehicle observations of deep sea red crabs, *Chaceon quinquedens*, off of the U.S. Continental Shelf, 226
Buoyancy, 121
Burger, Max M., see William J. Kuhns, 358
Busycon, 227
Bythites hollisi, 134

C

Calcium, 224, 232
Calcium carbonate ($CaCO_3$), 191
Calcium channels in identified neurons, 232
Calcium currents, 228
Calcium dynamics in the presynaptic terminals of barnacle photoreceptors, 228
Calcium oscillations, 224
Calcium waves, 222, 224
Calcium waves spread beneath the furrows of cleaving *Oryzias latipes* and *Xenopus laevis* eggs, 224
Calcium-dependence of inositol 1,4,5-trisphosphate-gated calcium-channels from endoplasmatic reticulum of cerebellum is bell-shaped, 228
Calibration of the effect of the air-sea interface on measurement of eelgrass and macroalgal density in Waquoit Bay, 226
Callaway, Joseph C., Nechama Lasser-Ross, Ann E. Stuart, and William N. Ross, Calcium dynamics in the presynaptic terminals of barnacle photoreceptors, 228
Campos de Carvalho, A. C., see D. C. Spray, 225
Cancer anthonyi, 201
Capsule morphology, 312
Carr, William E. S., see Henry G. Trapido-Rosenthal, 374
Carroll, David J., and Stephen C. Kempf, Laboratory culture of the aeolid nudibranch *Berghia verrucicornis* (Mollusca, Opisthobranchia): some aspects of its development and life history, 243
Case, James F., see Mark R. Bowlby, 340
Cell fusion, 220
Cell motility, 219–220
Centrosome, 264
Cephalopoda, 332
Cerebellum, 228
Chang, D. C., see Q. Zheng, 220
Chappell, Richard L., see Robert Paul Malchow, 231
Chemoreception, 234
Chemoreceptor cells, 234
Chemoreceptors, 374
Chemosensory adaptation, 234
Chemosensory response thresholds, 374
Cherksey, B. D., see A. L. Obaid, 232
Chimera, 279
Chromophore and polypeptide composition of *Aplysia* ink, The, 326
Chrystal, J., see I. Gillot, 224
Circadian rhythm, 231
Circadian rhythmicity of the crustacean hyperglycemic hormone (CHH) in the hemolymph of the crayfish, 351
Cleavage, 220, 224
Cohen, Larry B., see Chun X. Falk, 229
Cohen, Larry, Chun Xiao Falk, David Schiminovich, Jian-Young Wu, and Ray Falk, Cross-correlations in the spike activity of neurons in the *Aplysia* abdominal ganglion during the gill-withdrawal reflex, 229
Coleoptiles, 225
Collagen in the spicule organic matrix of the gorgonian *Leptogorgia virgulata*, 207
Communication, 87
Computer simulation, 223
Concentration-dependent tuning in lobster leg chemoreceptor cells, 234
Conch, 227
Consequences of the calcite skeletons of planktonic echinoderm larvae for orientation, swimming, and shape, 121
Coon, S. L., M. Walch, W. K. Fitt, R. M. Weiner, and D. B. Bonar, Ammonia induces settlement behavior in oyster larvae, 297
Copepod growth and survival, 113
Coralline algae, 304
Corals, 148
Crayfish, 351
Cross-correlations in the spike activity of neurons in the *Aplysia* abdominal ganglion during the gill-withdrawal reflex, 229
Crustacea, 233
Crustacean hyperglycemic hormone (CHH), 351
Crystallization, 191
Cuesta, Fabian C., see Richard P. Elinson, 163
Curtis, Lawrence, Parasitism and the movements of intertidal gastropod individuals, 105
Cushing response in bluefish, 233
Cyclic AMP-dependent phosphorylation of dynein heavy chains in *Mytilus edulis* sperm flagella, 226
Cytokeratin and desmoplakin analogues within an intracellular parasite, 237
Cytoskeletal elements in intracellular parasite, 237
Cytoskeleton, 220
Cytoskeleton/cytomatrix, 219

D

Dahlhoff, Elizabeth, Sabine Schneidemann, and George N. Somero, Pressure-temperature interactions on M_4-lactate dehydrogenases from hydrothermal vent fishes: evidence for adaptation to elevated temperatures by the zoarcid *Thermarces andersoni*, but not by the bythitid, *Bythites hollisi*, 134
Day-night cycle, 178
de Frescheville, Jacques, see Andre Toulmond, 366
Deegan, Linda A., see Heidi E. Geyer, 223; John T. Finn, 226; Kristen M. O'Brien, 223; Rebeka J. Rand, 223; and Robert Billard, 222
Deep sea hydrothermalism, 366
Del Pino, Eugenia M., see Richard P. Elinson, 163
Delaney, K. R., Y. Yamagata, D. W. Tank, P. Greenberg, and R. Llinas, Effects of synapsin I on synaptic facilitation at crayfish neuromuscular junction, 229
Demers, David, see E. L. Bearer, 219
Desperately seeking sex neurons: detection of projections in the male sex nerve of *Hirudo medicinalis* using nickel and horseradish peroxidase backfills, 232
Detailed observations on the migration of *Limulus* amoebocytes by video microscopy, 219
Determination of symmetry, 221
Determinative development, 221, 222
Development of a true ovoviviparous sea star, *Asterina pseudoexigua pacifca* Hayashi, 254
Development, terrestrial-breeding frogs, 163
Developmental biology, 220–222
Diffusion limitation and hyperoxic enhancement of oxygen consumption in zooxanthellate sea anemones, zoanthids, and corals, 148
Direct development, 254
Drag coefficients of swimming animals: effects of using different reference areas, 186
Drosophila, 77
DuBois, Arthur B., see Stephen H. Fox, 233
Dump Site 106, 226
Dynein, 226

E

Echinoderm, 121, 254, 264
Ecology and population biology, 222–224
Eelgrass, 223
Effects of cAMP-dependent protein kinase inhibitor on organelle movement in Y-1 adrenocortical tumor cells, 219
Effects of macroalgae on the abundance and diversity of free-swimming invertebrates in eelgrass beds of Waquoit Bay, MA, The, 223
Effects of macroalgae on the abundance of eelgrass (*Zostera marina*) in the Waquoit Bay Estuary, The, 223

Effects of stimulus duration on the chemoreceptor responses of the medial antennule of *Homarus americanus*, The, 234
Effects of synapsin I on synaptic facilitation at crayfish neuromuscular junction, 229
Effects of tumor promoters (okadaic acid, TPA) and anticarcinogens (nicotinamide, sarcophytol A) on *Arabacia* development, 221
Efflux of amino acids from the olfactory organ of the spiny lobster: biochemical measurements and physiological effects, The, 374
Egg capsules, 312
Egg mortality, 201
Ehrlich, Barbara E., see Ilya B. Bezprozvanny, 228; and Susan R. Barry, 228
Eichhorn, Peter, see Richard P. Elinson, 163
Elasmobranch, 224
Elasmobranch eye lens: actin and UV radiation, 220
Electrofusion, 220
Eleutherodactylus coqui, 163
Elinson, Richard P., Eugenia M. Del Pino, Daniel S. Townsend, Fabian C. Cuesta, and Peter Eichhorn, A practical guide to the developmental biology of terrestrial-breeding frogs, 163
Embryo culture, terrestrial-breeding frogs, 163
Encapsulation, 312
Euprymna scolopes light organ, 332
Evolution, 87
Extracellular hemoglobins of hydrothermal vent annelids: structural and functional characteristics in three alvinellid species, 366
Extracellular matrix components, 220
Extraction of a vanadium-binding substance (vanadobin) from the blood cells of several ascidian species, 140

F

Facilitation, 229
Falk, Chun X., Win Watson III, Jim Trimarchi, Jian-young Wu, and Larry B. Cohen, Preliminary optical measurements on the *Melibe leonina* buccal ganglion, 229
Falk, Chun Xiao, see Larry Cohen, 229
Falk, Ray, see Larry Cohen, 229
Feeding and predation rates of winter flounder, *Pseudopleuronectes americanus*, and blue crabs, 222
Feinman, Richard D., Hester Kothals Altes, Sam Kingston, Charles I. Abramson, and Robin R. Forman, Lever-press conditioning in the crab. Green crabs perform well on fixed ration schedules, but can they count? 233
Fertilization, 183
Fertilization and early development, 224–226
Field investigation, 178
Fini, M. E., see M. L. Pardue, 77
Finn, Jack (John) T., see Heidi E. Geyer, 223; Kristen M. O'Brien, 223; Rebeka J. Rand, 223; and Robert Billard, 222
Finn, John T., Linda A. Deegan, and David Paton, Calibration of the effect of the air-sea interface on measurement of eelgrass and macroalgal density in Waquoit Bay, 226
Firefly unisex flash controls, 87
Fish, 223
Fitt, W. K., see S. L. Coon, 297
Flagella, 226
Flatworm, 220
Fluck, Richard A., see Andrew L. Miller, 224
Fluorescent dyes, 230
Forman, Robin R., see Richard D. Feinman, 233
Fox, Stephen H., Cristopher S. Olgilvy, and Arthur B. DuBois, Search for the biological stimulus of the Cushing response in bluefish, 233
Frenkel, Krystyna, Hirota Fujiki, Albert Grossman, and Walter Troll, Effects of tumor promoters (okadaic acid, TPA) and anticarcinogens (nicotinamide, sarcophytol A) on *Arabacia* development, 221
Frogs, terrestrial-breeding, 163
FTX, an HPLC-purified fraction of funnel web spider venom, blocks calcium channels required for normal release in peptidergic nerve terminals of mammals: optical measurements with and without voltage-sensitive dyes, 232
Fujiki, Hirota, see Krystyna Frenkel, 221
Functional autonomy of land and sea orientation systems in sea turtle hatchlings, 214
Functional morphology, 312
FURA, 228
Fuseler, John, see Earl Weidner, 237

G

GABA-induced currents of internal horizontal cells of the skate retina, 231
Galivan, John, see Robert MacColl, 326
Gap junction channels in marine embryos: comparison of properties in late blastulae of squid and skate, 225
Garbe, J. C., see M. L. Pardue, 77
Gas exchange, 148
Gastropods, 105, 312
Gastrotheca riobambae, 163
Geographic variation in naupliar growth and survival in a harpacticoid copepod, 113
Gerardo, H. F., Rainer Voigt, and Jelle Atema, Concentration-dependent tuning in lobster leg chemoreceptor cells, 234
Germinal vesicle, 224
Geyer, Heidi E., Linda A. Deegan, Jack T. Finn, and Suzanne G. Ayvazian, The effects of macroalgae on the abundance of eelgrass (*Zostera marina*) in the Waquoit Bay Estuary, 223
Gigantic germinal vesicles of elasmobranchs, The, 224
Gill withdrawal, 229
Gilland, E., and R. Baker, Segmental location of cranial nerve roots and motor nuclei in *Squalus acanthias*, 230
Gillot, I., J. Chrystal, L. F. Jaffe, and W. M. Kühtreiberg, Where does the calcium lost by fertilizing *Arbacia* eggs go? 224
Gleeson, Richard A., see Henry G. Trapido-Rosenthal, 374
Glycine, 374
Glynn, Paul, see Robert Paul Malchow, 231
Goldberg, Walter M., Ken R. Grange, George T. Taylor, and Alicia L. Zuniga, The structure of sweeper tentacles in the black coral *Antipathes fiordensis*, 96
Goldman, A., see C. D. Leidigh, 224
Goldman, R. D., see C. D. Leidigh, 224
Gomes, George, Rainer Voight, and Jelle Atema, The effects of stimulus duration on the chemoreceptor responses of the medial antennule of *Homarus americanus*, 234
Gorgonian, 207
Gossypol-binding proteins from marine species, 226
Grafting, 279
Grange, Ken R., see Walter M. Goldberg, 96
Gravitational stability, 121
Greengard, P., see K. R. Delaney, 229
Greenough Pond project planning: Yarmouthport, Massachusetts; acid water and macro flora is the base line of H. Sverdop and P. Warfingers' chain of response decision tree, 227
Grossman, Albert, see Krystyna Frenkel, 221
Growth, size, and enzymes in *Artemia*, 287
Guard-Friar, Deborah, see Robert MacColl, 326
Gunthorpe, M. E., C. S. Sikes, and A. P. Wheeler, Promotion and inhibition of calcium carbonate crystallization *in vitro* by matrix protein from blue crab exoskeleton, 191
Gynaecotyla adunca, 105

H

Halichoerus grypus at Monomoy Island, winter of 1988–1989: photogrammetry showing shift east and north of traditional birthing sites, 228
Halichoerus grypus, births, location change, 228
Harpacticoid, 113
Heat shock, 77
Heath, Mark, see Alan M. Kuzirian, 227

Hemigrapsus spp., 312
Hemolymph, 351
Hermaphroditism, 254
Hermissenda, 227, 232
Hermit crabs, 235
Herzog, Erik, and Robert Barlow Jr., The *Limulus*-eye view of the world, 230
Heteractis crispa, 148
Hirose, Hisayoshi, see Hitoshi Michibata, 140
Hirudo medicalis, 232
Histocompatibility, 279
Hoffman, Daniela E., see Ilene M. Kaplan, 227
Hogan, N. C., see M. L. Pardue, 77
Homola, Ellen, see Hans Laufer, 221
Hoploplana, 221
Horizontal cells, 231
Horseshoe crab, 223
Host-symbiont relationship, 201
Hsr-omega, a novel gene encoded by a *Drosophila* heat shock puff, 77
Husbandry, amphibian, 163
Hydrodynamics, 148, 186
Hydrothermal vents, 134
Hyperoxia and respiration in anthozoans, 148

I

Idotea, 186, 312
Ikai, Atsushi, see Peter B. Armstrong, 233
Ilan, Micha, and Yossi Loya, Ontogenetic variation in sponge histocompatibility responses, 279
Ilyanassa, 105, 222
Inactivation of squid rhodopsin in the absence of phosphorylation, 230
Induction of metamorphosis of larvae of the green sea urchin, *Strongylocentrotus droebachiensis*, by coralline red algae, 304
Influence of macroalgae in eelgrass beds on finfish abundance and dissolved oxygen in Waquoit Bay, 223
Inhibition, 191
Inland culture of a nudibranch, 243
Inositol 1,4,5-trisphosphate, 228
Insect neurobiology, 87
Intentional catch (*Busycon*) and unintentional catch (*Hoploplana*) by fishermen and the question of seafood inspection, 227
Interommatidial angle, 230
Intertidal ecology, 105
Intracranial pressure, 233
Intraspecific variation in egg capsule morphology, 312
Invertebrate, 223
Isomate, 234
Isopod, 186

J

Jaffe, L. F., see I. Gillot, 224
Jaffe, Lionel F., see Andrew L. Miller, 224; and Richard M. Sanger, 225
Jaffe, Lionel F., The path of calcium in fertilization and other endogenous oscillations: a unifying view, 224
Jonasdottir, Sigrun H., see Darcy J. Lonsdale, 113
Jouin, Claude, see Andre Toulmond, 366

K

Kahana, Alon, Phyllis R. Robinson, and John E. Lisman, Inactivation of squid rhodopsin in the absence of phosphorylation, 230
Kallen, Janine L., S. L. Abrahamse, and F. van Herp, Circadian rhythmicity of the crustacean hyperglycemic hormone (CHH) in the hemolymph of the crayfish, 351
Kanamori, Kan, see Hitoshi Michibata, 140
Kano, Yasuo T., see Miéko Komatsu, 254
Kaplan, Ilene M., Barbara C. Boyer, and Daniela E. Hoffman, Intentional catch (*Busycon*) and unintentional catch (*Hoploplana*) by fisherman and the question of seafood inspection, 227
Karplus, Eric, see Richard M. Sanger, 225
Kempf, Stephen C., see David J. Carroll, 243
Kim, N. H., see C. D. Leidigh, 224; and D. C. Spray, 225
Kinase, 219
Kingsley, Roni J., Mari Tsuzaki, Norimitsu Watabe, and Gerald L. Mechanic, Collagen in the spicule organic matrix of the gorgonian *Leptogorgia virgulata*, 207
Kingston, Sam, see Richard D. Feinman, 233
Koide, S. S., see Hiroshi Ueno, 226
Komatsu, Miéko, Yasuo T. Kano, and Chitaru Oguro, Development of a true ovoviviparous sea star, *Asterina pseudoexigua pacifica* Hayashi, 254
Komuro, H., A. L. Obaid, S. S. Kumar, and B. M. Salzberg, Slices of mouse suprachiasmatic nucleus with attached optic nerve: recording of glutaminergic and GABA-ergic postsynaptic potentials using a voltage-sensitive dye, 231
Komuro, H., see A. L. Obaid, 232
Korthals Altes, Hester, see Richard D. Feinman, 233
Kuhns, William J., Gradimir Misevic, and Max M. Burger, Biochemical and functional effects of sulfate restriction in the marine sponge, *Microciona prolifera*, 358
Kühtreiber, W. M., see I. Gillot, 224
Kumar, S. S., see A. L. Obaid, 232; and H. Komuro, 231
Kuris, Armand, see Hans Laufer, 221
Kuzirian, Alan M., Catherine T. Tamse, and Mark Heath, Ozonation of natural seawater affects the embryology of *Hermissenda crassicornis*, 227
Kuzirian, Alan, see Ebenezer Yamoah, 232

L

Laboratory culture of the aeolid nudibranch *Berghia verrucicornis* (Mollusca, Opisthobranchia): some aspects of its development and life history, 243
Langford, George M., Edward E. Leonard, Dieter G. Weiss, and Sandra A. Murray, Effects of cAMP-dependent protein kinase inhibitor on organelle movement in Y-1 adrenocortical tumor cells, 219
Larvae, 297, 304
Larval
  aggregation, 279
  development, 243
  fusion, 279
  skeletons and orientation, 121
Lasser-Ross, Nechama, see Joseph C. Callaway, 228
Laufer, Hans, Ellen Homola, Armand M. Kuris, and Amir Sagi, Morphology and reproductive tract development in winter and summer populations of the male spider crab *Libinia emarginata*: a proposed life history and regulatory mechanism, 221
LDHs of hydrothermal vent fishes, 134
Learning, 233
Leidhigh, C., see D. C. Spray, 225
Leidigh, C. D., N. H. Kim, R. D. Goldman, A. Goldman, M. V. L. Bennett, and G. D. Pappas, The gigantic germinal vesciles of elasmobranchs, 224
Lens, 220
Leonard, Edward E., see George M. Langford, 219
Leptogorgia virgulata, 207
Leucandra, 272
Lever-press conditioning in the crab. Green crabs perform well on fixed ration schedules, but can they count? 233
Libinia emarginata, 221
Light organ, *Euprymna*, 332
Limulus, 219, 233
Limulus-eye view of the world, The, 230
Linn, J.-W., see A. L. Obaid, 232
Lisman, John E., see Alon Kahana, 230
Lithothamnion glaciale, 304

Llinas, R., see A. L. Obaid, 232; and K. R. Delaney, 229
Lobster, 234, 374
Lohmann, Kenneth J., Michael Salmon, and Jeanette Wyneken, Functional autonomy of land and sea orientation systems in sea turtle hatchlings, 214
Lonsdale, Darcy J., and Sigrun H. Jonasdottir, Geographic variation in naupliar growth and survival in a harpacticoid copepod, 113
Lowe, Kris C., see Seymour Zigman, 220
Loya, Yossi, see Micha Ilan, 279
Lynch, Laura, see Robert Billard, 222
Lyoluminescence, 232
Lyosomes, 219

M

Macagno, Eduardo, see Michael Nitabach, 232
MacColl, Robert, John Galivan, Donald S. Berns, Zenia Nimec, Deborah Guard-Friar, and David Wagoner, The chromophore and polypeptide composition of *Aplysia* ink, 326
Macroalgae, 223
Macromere control of early development in the polyclad flatworm, *Hoploplana*, 221
Macromere deletions, 221
Malchow, Robert Paul, Richard L. Chappell, Paul Glynn, and Harris Ripps, GABA-induced currents of internal horizontal cells of the skate retina, 231
Mangel, Walter F., see Peter B. Armstrong, 233
Marine embryo coupling, 225
Marine policy, 227
Maruyama, Yoshihiko, K., Roles of the polar cytoplasmic region in meiotic divisions in oocytes of the sea cucumber *Holothuria leucospilota*, 264
Maximal enzyme activities, 287
McFall-Ngai, Margaret, and Mary K. Montgomery, The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae), 332
McLaughlin, Jane A., see Andrew L. Miller, 224
Mechanic, Gerald L., see Roni J. Kingsley, 207
Meiosis, 264
Meiotic spindle organizing centers, 264
Melibe, 229
Metalloproteinases of the developing sea urchin embryo, 221
Metamorphosis, 243, 304
Methyl farnesoate, 221
Michibata, Hitoshi, Hisayoshi Hirose, Kiyomi Sugiyama, Yukari Ookubo, and Kan Kanamori, Extraction of a vanadium-binding substance (vanadobin) from the blood cells of several ascidian species, 140
Microciona prolifera, 358
Micromere deletions, 222
Microscale fluid dynamics, 234
Microsporidian cytoskeletal elements, 237
Migration, 214
Miller, Andrew L., Richard A. Fluck, Jane A. McLaughlin, and Lionel F. Jaffe, Calcium waves spread beneath the furrows of cleaving *Oryzias latipes* and *Xenopus laevis* eggs, 224
Mineral induction, 191
Misevic, Gradimir, see William J. Kuhns, 358
Montgomery, Mary K., see Margaret McFall-Ngai, 332
Moore, Paul A., see Adele Pile, 234; Jelle Atems, 234; and Nat Scholz, 235
Moreno, A. P., see D. C. Spray, 225
Moreno, Alonso P., and David C. Spray, Sea urchin embryos: suitability for exogenous expression of gap junction channels, 225
Morphogenesis of ascidian ampullae and polarized movements of tunic extracellular matrix components along ampullae, 220
Morphology and reproductive tract development in winter and summar populations of the male spider crab *Libinia emarginata*: a proposed life history and regulatory mechanism, 221
Mosaic development, 220
Motility, 219
Motor nuclei, 230
mRNA expression, 225
Murray, Sandra A., see George M. Langford, 219

N

Naupliar growth and survival, 113
Nematocyst, 96
Nemoto, Shin-Ichi, see Mitsuki Yoneda, 183
Nerve
  backfills, 232
  sealing, 229
  terminals, 232
Neuromeres, 230
Neurons, 232
Niche displacement, models of, 223
Nickel chloride, 232
Nimec, Zenia, see Robert MacColl, 326
Nitabach, Michael, and Eduardo Macagno, Desperately seeking sex neurons: detection of projections in the male sex nerve of *Hirudo medicinalis* using nickel and horseradish peroxidase backfills, 232
Nocturnal emergence activity rhythm in the cumacean *Dimorphostylis asiatica* (Crustacea), 178
Nucella emarginata, 312
Nuclear
  envelope, 224
  fusion, 183
  RNA, 77
Nudibranch, 243

O

O'Brien, Kristin M., Linda A. Deegan, John T. Finn, and Suzanne G. Ayvazian, The effects of macroalgae on the abundance and diversity of free-swimming invertebrates in eelgrass beds of Waquoit Bay, MA, 223
Obaid, A. L., H. Komuro, S. S. Kumar, M. Sugimori, J.-W. Lin, B. D. Cherksey, R. Llinas, and B. M. Salzberg, FTX, an HPLC-purified fraction of funnel web spider venom, blocks calcium channels required for normal release in peptidergic nerve terminals of mammals: optical measurements with and without voltage-sensitive dyes, 232
Obaid, A. L., see H. Komuro, 231
Occurrence of partial nuclei in eggs of the sand dollar, *Clypeaster japonicus*, 183
Odor flow within normal cavity, 234
Odor plumes, 235
Odorant behavior, 374
Ogilvy, Christopher S., see Stephen H. Fox, 233
Oguro, Chitaru, see Mieko Komatsu, 254
Olfaction, 374
Olfactory sensilla, 374
Ontogenetic variation in sponge histocompatibility responses, 279
Oocytes, sea cucumber, 264
Ookubo, Yukari, see Hitoshi Michibata, 140
Operant conditioning, 233
Optical, 229
Optical recording, 232
Optics, 230
Organic matrix, 191
Orientation, 214, 235
Ornithine decarboxylase exhibits negative thermal modulation in the sea star *Asterias vulgaris*: potential regulatory role during temperature-dependent testicular growth, 159
Osmolytes, 374
Overstreet, Robin M., see Earl Weidner, 237
Oyster, 297
Ozonation of natural seawater affects the embryology of *Hermissenda crassicornis*, 227

P

Palythoa tuberculosa, 148
Panulirus argus, 374
Pappas, G. D., see C. D. Leidigh, 224; and D. C. Spray, 225
Paralvinella, 366
Paramecium, 228
Parasitism and the movements of intertidal gastropod individuals, 105
Pardue, M. L., W. G. Bendena, M. E. Fini, J. C. Garbe, N. C. Hogan, and K. L. Traverse, *Hsr-omega*, a novel gene encoded by a *Drosophila* heat shock puff, 77
Partial nuclei in sand dollar eggs, 183
Passive orientation, 121
Path of calcium in fertilization and other endogenous oscillations: a unifying view, The, 224
Paton, David, Greenough Pond project planning: Yarmouthport, Massachusetts; acid water and macro flora is the base line of H. Sverdop and P. Warfingers' chain of response decision tree, 227
Paton, David, *Halichoerus grypus* at Monomoy Island, winter of 1988–1989: photogrammetry showing shift east and north of traditional birthing sites, 228
Paton, David, see John T. Finn, 226
Patterns of stimulated bioluminescence in two pyrosomes (Tunicata; Pyrosomatidae), 340
Pearce, Christopher M., and Robert E. Scheibling, Induction of metamorphosis of larvae of the green sea urchin, *Strongylocentrotus droebachiensis*, by coralline red algae, 304
Pennington, J. Timothy, and Richard R. Strathmann, Consequences of the calcite skeletons of planktonic echinoderm larvae for orientation, swimming, and shape, 121
Phosphorylation, 226, 230
Photic stimulation, 340
Phyllodiscus semoni, 148
Pile, Adele, Paul Moore, and Jelle Atema, Three-dimensional odor flow within the nasal cavity of the bullhead catfish, 234
Pluteus, 121
Poeciloogony, 243
Polar body formation, 264
Polarity, 222
Polarity and meiosis in oocytes, 264
Polyamines, 159
Polymorphic males, 221
Polytene chromosomes, 77
Pomatumus saltatrix, 233
Pond, acid, management, 227
Practical guide to the developmental biology of terrestrial-breeding frogs, 163
Predation on egg capsules, 312
Predation rates, 222
Preliminary optical measurements on the *Melibe leonina* buccal ganglion, 229–230
Pressure-temperature interactions on M_4-lactate dehydrogenases from hydrothermal vent fishes: evidence for adaptation to elevated temperatures by the zoarcid *Thermarces andersoni*, but not by the bythitid, *Bythites hollisi*, 134
Presynaptic terminal, 228
Prior, G., see R. E. Stephens, 226
Promotion and inhibition of calcium carbonate crystallization *in vitro* by matrix protein from blue crab exoskeleton, 191
Protein
  content, 287
  synthesis, 77
Proteolytic enzymes, 221
Protistan cytokeratin and desmoplakin analogues, 237
Protopalythoa sp., 148
Pyrosome bioluminescence, 340

Q

Quigley, J., R. S. Braithwaite, and P. Armstrong, Metalloproteinases of the developing sea urchin embryo, 221
Quigley, James P., see Peter B. Armstrong, 233

R

Rafferty, Nancy S., see Seymour Zigman, 220
Raja, 231
Rand, Rebeka J., Linda A. Deegan, John T. Finn, and Suzanne G. Ayvazian, Influence of macroalgae in eelgrass beds on finfish abundance and dissolved oxygen in Waquoit Bay, 223
Rawlings, Timothy A., Associations between egg capsule morphology and predation among populations of the marine gastropod, *Nucella emarginata*, 312
Recruitment, 297
Red algae, 326
Red crab, 226
Relationship between body size, growth rate, and maximal enzyme activities in the brine shrimp, *Artemia franciscana*, 287
Relationship between flow and chemical signals in providing directional cues for chemically orientating hermit crabs, The, 235
Released CHH in crayfish, 351
Reorganization of cytoskeleton during cell fusion induced by electric field, 220
Repetitive calcium waves in the fertilized ascidian egg are intitiated in the vegetal hemisphere by a cortical pacemaker, The, 222
Reproductive ecology, 312
Reproductive system regulation, 221
Reptile, 214
Reynolds, G. T., Lyoluminescence, 232
Rhizophydium littoreum on the eggs of *Cancer anthonyi*: parasite or saprobe? 201
Rhodopsin, squid, 230
Rhythmic behavior in the field, 178
Ripps, Harris, see Robert Paul Malchow, 231
Robinson, Phyllis R., see Alon Kahana, 230
Roff, John C., see John A. Berges, 287
Roles of the polar cytoplasmic region in meiotic divisions in oocytes of the sea cucumber, *Holothuria leucospilota*, 264
Ross, William N., see Joseph C. Callaway, 228
Roy, J., see Stephen A. Watts, 159

S

S.E.M. observations of early cleavage in *Hoploplana inquilina*, 220
Sagi, Amir, see Hans Laufer, 221
Salmon, Michael, see Kenneth J. Lohmann, 214
Salzberg, B. M., see A. L. Obaid, 232; and H. Komuro, 231
Sand dollar egg, 183
Sanger, Richard A., Eric Karplus, and Lionel F. Jaffe, An aerial vibrating probe, 225
Scemes, E., see D. C. Spray, 225
Scheibling, Robert E., see Christopher M. Pearce, 304
Schiminovich, David, see Larry Cohen, 229
Schneidemann, Sabine, see Elizabeth Dahlhoff, 134
Scholz, Nat, Paul A. Moore, and Jaffe Atema, The relationship between flow and chemical signals in providing directional cues for chemically orientating hermit crabs, 235
Sea anemones, 148
Sea cucumber, 264
Sea turtle wave orientation, 214
Sea urchin, 224, 225, 304
Sea urchin embryos: suitability for exogenous expression of gap junction channels, 225
Seafood inspection, 227
Search for the biological stimulus of the Cushing response in bluefish, 233
Seasonal variation, 178
Segal, Sheldon J., see Hiroshi Ueno, 226
Segmental location of cranial nerve roots and motor nuclei in *Squalus acanthias*, 230
Self and non-self recognition in a calcereous sponge, *Leucandra abratsbo*, 272
Settlement, 304
Settlement behavior, 297

Shell disease, 226

Shick, J. Malcolm, Diffusion limitation and hyperoxic enhancement of oxygen consumption in zooxanthellate sea anemones, zoanthids, and corals, 148

Shields, Jeffrey D., *Rhizophydium littoreum* on the eggs of *Cancer anthonyi*: parasite or saprobe? 201

Sikes, C. S., see M. E. Gunthorpe, 191

Single and multiple micromere deletions in first quartet embryos of *Ilyanassa obsoleta*, 222

Single channels, 225

Sinking, 121

Size-scaling, 287

Skate retina, 231

Skeleton, 121

Slices of mouse suprachiasmatic nucleus with attached optic nerve: recording of glutaminergic and GABA-eric postsynaptic potentials using a voltage-sensitive dye, 231

Slitine, Fouzia el Idrissi, see Andre Toulmond, 366

Somero, George N., see Elizabeth Dahlhoff, 134

Speksnijder, J. E., The repetitive calcium waves in the fertilized ascidian egg are intitiated in the vegetal hemisphere by a cortical pacemaker, 222

Spermatogenesis, 159

Spicule matrices, 207

Spirocyst, 96

Sponge, 272, 358

Sponge histocompatibility responses, 279

Spray, D. C., A. C. Campos de Carvalho, A. P. Moreno, E. Scemes, C. Leidhigh, N. H. Kim, G. D. Pappas, and M. V. L. Bennett, Gap junction channels in marine embryos: comparison of properties in late blastulae of squid and skate, 225

Spray, David C., see Alonso P. Moreno, 225

Squid, 332
  giant axon, 229
  rhodopsin, 230

Stephens, R. E., and G. Prior, Cyclic AMP-dependent phosphorylation of dynein heavy chains in *Mytilus edulis* sperm flagella, 226

Stochastic and deterministic models of niche displacement, 223

Stockbridge, Norman, see Ebenezer Yamoah, 232

Strathmann, Richard R., see J. Timothy Pennington, 121

Strongylocentrotus droebachiensis, 304

Strout, M., see E. L. Bearer, 219

Structure of alpha-2 macroglobulin from the horseshoe crab, 233

Structure of sweeper tentacles in the black coral *Antipathes fiordensis*, The, 96

Stuart, Ann E., see Joseph C. Callaway, 228

Stylophora pistillata, 148

Submersible vehicle observations of deep sea red crabs, *Chaceon quinquedens*, off of the U.S. continental shelf, 226

Sugimori, M., see A. L. Obaid, 232

Sugiyama, Kiyomi, see Hitoshi Michibata, 140

Sulfate restriction in marine sponge, 358

Suprachiasmatic nucleus, 231

Sweeper tentacles, 96

Sweet, Hyla C., and Barbara C. Boyer, Single and multiple micromere deletions in first quartet embryos of *Ilyanassa obsoleta*, 222

Sweet, Hyla C., see John M. Arnold, 220

Swimming, 121, 186

Symbiosis, 201

Synapsin I, 229

Synaptic transmission, 229

T

Tamse, Catherine T., see Alan M. Kuzirian, 227

Tank, D. W., see K. R. Delaney, 229

Taurine, 374

Taylor, George T., see Walter M. Goldberg, 96

Tedeschi, Bruce, see Earl Weidner, 237

Temperature adaptation, 159

Terrestrial-breeding frogs, 163

Thermal modulation of ODC, 159

Thermarces andersoni, 134

Three-dimensional odor flow within the nasal cavity of the bullhead catfish, 234

Tidal cycle, 178

Toulmond, Andre, Fouzia el Idrissi Slitine, Jacques de Frescheville, and Claude Jouin, Extracellular hemoglobins of hydrothermal vent annelids: structural and fuctional characteristics in three alvinellid species, 366

Townsend, Daniel S., see Richard P. Elinson, 163

Trapido-Rosenthal, Henry G., Richard A. Gleeson, and William E. S. Carr, The efflux of amino acids from the olfactory organ of the spiny lobster: biochemical measurements and physiological effects, 374

Traverse, K. L., see M. L. Pardue, 77

Trematod parasitism, 105

Trimarchi, Jim, see Chun X. Falk, 229–230

Troll, Walter, see Krystyna Frenkel, 221

Tsuzaki, Mari, see Roni J. Kinsley, 207

Tumor promoters, 221

Tunicate, 140

Tuning, 234

U

Ueno, Hiroshi, Sheldon J. Segal, and S. S. Koide, Gossypol-binding proteins from marine species, 226

Ultrastructure, 96

Unisex flash controls in dialog fireflies, 87

UV radiation, 220

V

van Egeraat, J. M., and J. P. Wikswo Jr., Application of a magnetic current probe to map axial inhomogeneities in a squid giant axon, 229

van Herp, F., see Janine L. Kallen, 351

Van Holde, Kensal E., see Peter B. Armstrong, 233

Vanadobin from ascidians, 140

Vibrating probe, 225

Vibrio fischeri, 332

Viviparity, 254

Voigt, Rainer, see George Gomez, 234; and H. F. Gerardo, 234

Voltage-sensitive dyes, 231

W

Wagoner, David, see Robert MacColl, 326

Walch, M., see S. L. Coon, 297

Walker, C. W., see Stephen A. Watts, 159

Waquoit Bay, 223

Watabe, Norimitsu, see Roni J. Kinsley, 207

Water flow, 148

Watras, James, see Ilya B. Bezprozvanny, 228

Watson, Win, III, see Chun X. Falk, 229

Watts, Stephen A., J. Roy, and C. W. Walker, Ornithine decarboxylase exhibits negative thermal modulation in the sea star *Asterias vulgaris*: potential regulatory role during temperature-dependent testicular growth, 159

Wave, 214

Weidner, Earl, Robin M. Overstreet, Bruce Tedeschi, and John Fuseler, Cytokeratin and desmoplakin analogues within an intracellular parasite, 237

Weiner, R. M., see S. L. Coon, 297

Weiss, Dieter G., see George M. Langford, 219

Wheeler, A. P., see M. E. Gunthorpe, 191

Where does the calcium lost by fertilizing *Arbacia* eggs go? 224

Widder, Edith A., see Mark R. Bowlby, 340

Wikswo, J. P., Jr., see J. M. van Egeraat, 229

Winter flounder, 222

WU, JIAN-YOUNG, see Chun X. Falk, 229–230; and Larry Cohen, 229
WYNEKEN, JEANETTE, see Kenneth J. Lohmann, 214

Y

YAMAGATA, Y., see K. R. Delaney, 229
YAMOAH, EBENEZER, NORMAN STOCKBRIDGE, AND ALAN KUZIRIAN, Calcium channels in identified neurons, 232
YONEDA, MITSUKI, AND SHIN-ICHI NEMOTO, Occurrence of partial nuclei in eggs of the sand dollar, *Clypeaster japonicus*, 183
YOSHIDA, M., see T. Akiyama, 178

Z

ZHENG, Q., AND D. C. CHANG, Reorganization of cytoskeleton during cell fusion induced by electric field, 220
ZIGMAN, SEYMOUR, NANCY S. RAFFERTY, AND KRIS C. LOWE, Elasmobranch eye lens: actin and UV radiation, 220
Zoanthids, 148
Zoanthus sociatus, 148
Zooxanthellae, 148
Zostera marina, 223
ZUNIGA, ALICIA L., see Walter M. Goldberg, 96

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CONTENTS

CELL STRUCTURE

Weidner, Earl, Robin M. Overstreet, Bruce Tedeschi, and John Fuseler
Cytokeratin and desmoplakin analogues within an intracellular parasite 237

DEVELOPMENT AND REPRODUCTION

Carroll, David J., and Stephen C. Kempf
Laboratory culture of the aeolid nudibranch *Berghia verrucicornis* (Mollusca, Opisthobranchia): some aspects of its development and life history 243

Komatsu, Miéko, Yasuo T. Kano, and Chitaru Oguro
Development of a true ovoviviparous sea star, *Asterina pseudoexigua pacifica* Hayashi 254

Maruyama, Yoshihiko K.
Roles of the polar cytoplasmic region in meiotic divisions in oocytes of the sea cucumber, *Holothuria leucospilota* 264

ECOLOGY AND EVOLUTION

Amano, Shigetoyo
Self and non-self recognition in a calcareous sponge, *Leucandra abratsbo* 272

Ilan, Micha, and Yossi Loya
Ontogenetic variation in sponge histocompatibility responses 279

Berges, John A., John C. Roff, and James S. Ballantyne
Relationship between body size, growth rate, and maximal enzyme activities in the brine shrimp, *Artemia franciscana* 287

Coon, S. L., M. Walch, W. K. Fitt, R. M. Weiner, and D. B. Bonar
Ammonia induces settlement behavior in oyster larvae 297

Pearce, Christopher M., and Robert E. Scheibling
Induction of metamorphosis of larvae of the green sea urchin, *Strongylocentrotus droebachiensis,* by coralline red algae 304

Rawlings, Timothy A.
Associations between egg capsule morphology and predation among populations of the marine gastropod, *Nucella emarginata* 312

GENERAL BIOLOGY

MacColl, Robert, John Galivan, Donald S. Berns, Zenia Nimec, Deborah Guard-Friar, and David Wagoner
The chromophore and polypeptide composition of *Aplysia* ink 326

McFall-Ngai, Margaret, and Mary K. Montgomery
The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda:Sepiolidae) 332

PHYSIOLOGY

Bowlby, Mark R., Edith A. Widder, and James F. Case
Patterns of stimulated bioluminescence in two pyrosomes (Tunicata: Pyrosomatidae) 340

Kallen, Janine L., S. L. Abrahamse, and F. Van Herp
Circadian rhythmicity of the crustacean hyperglycemic hormone (CHH) in the hemolymph of the crayfish 351

Kuhns, William J., Gradimir Misevic, and Max M. Burger
Biochemical and functional effects of sulfate restriction in the marine sponge, *Microciona prolifera* . . . 358

Toulmond, Andre, Fouzia el Idrissi Slitine, Jacques de Frescheville, and Claude Jouin
Extracellular hemoglobins of hydrothermal vent annelids: structural and functional characteristics in three alvinellid species 366

Trapido-Rosenthal, Henry G., Richard A. Gleeson, and William E. S. Carr
The efflux of amino acids from the olfactory organ of the spiny lobster: biochemical measurements and physiological effects 374

Index to Volume 179 383

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